DERMATAN SULFATE: A NEW CONCEPT IN ANTITHROMBOTIC THERAPY

by

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ABSTRACT

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This study was undertaken to explore the hypothesis that dermatan sulfate, which catalyses thrombin inhibition by heparin cofactor II, prevents thrombosis more effectively than heparin, which catalyses thrombin inhibition by antithrombin III. The rationale for this hypothesis is based upon three general observations. First, higher doses of heparin are required for the treatment of thrombus growth than are required for the prevention of thrombus formation. Second, thrombin bound to fibrin or to vessel wall components in vitro is resistant to inhibition by heparin/antithrombin III. Third, preliminary evidence indicates that the catalysis of thrombin inhibition by antithrombin III-independent inhibitors may be more effective than heparin/antithrombin III. The results of this present study support the above hypothesis and demonstrated that dermatan sulfate, a glycosaminoglycan which catalyses thrombin inhibition by heparin cofactor II, inhibited i) thrombus formation, ii) thrombus growth, and iii) acted synergistically with tPA to enhance thrombolysis, more effectively than heparin. These effects were achieved with
minimal detrimental side-effects. These results indicate that dermatan sulfate is an effective antithrombotic agent and support the conclusion that the catalysis of thrombin inhibition by dermatan sulfate/heparin cofactor II provides a more effective pathway for preventing thrombus formation and thrombus growth than the catalysis of thrombin inhibition by heparin/antithrombin III.
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CHAPTER 1: INTRODUCTION
Heparin is the most commonly used anticoagulant both for the treatment and prevention of venous thrombosis, and as an adjunct with antiplatelet and fibrinolytic drugs for the treatment of arterial thrombotic complications. The rationale for the use of heparin is, that heparin catalyses factor Xa and thrombin inhibition by antithrombin III, thereby preventing further thrombin generation and subsequent thrombus formation and/or thrombus growth.

There are, however, a number of unresolved issues concerning heparin therapy which suggest that our understanding of the mechanisms by which heparin achieves its antithrombotic effects are not entirely clear. First, it is unclear whether the antithrombotic effect of heparin (and a number of its low molecular weight fractions) is best achieved by catalysing factor Xa inhibition or by catalysing thrombin inhibition. Second, heparin can also catalyse the inhibition of thrombin by another cofactor, heparin cofactor II. While it has been demonstrated that the catalysis of thrombin inhibition by antithrombin III is an effective route for achieving an antithrombotic effect, recent evidence suggests that catalysis of thrombin inhibition by heparin cofactor II may also be effective in vivo. Third, higher doses of heparin are required for the treatment of venous thrombosis than are required for the prevention of venous thrombosis. While the explanation for this difference in dose requirements is
unclear, it has been suggested that thrombin is resistant to inhibition when it is bound to fibrin, i.e. to an existing thrombus. And finally, while heparin is a useful antithrombotic agent, its use is also associated with significant bleeding. The mechanism underlying this side-effect, relative to its antithrombotic effect is not clearly understood.

Studies in our laboratory investigating the relative importance of factor Xa and thrombin inhibition in the prevention of thrombus formation, have resulted in a number of observations which provide insight into these issues. First, it was demonstrated that another glycosaminoglycan, dermatan sulfate, was as effective as heparin in preventing thrombus formation. Dermatan sulfate, unlike heparin, catalysed thrombin inhibition by heparin cofactor II, not by antithrombin III. Second, the antithrombotic effect of dermatan sulfate was achieved without catalysing the inhibition of factor Xa. These two observations indicated for the first time that heparin cofactor II may be an important endogenous inhibitor of thrombin. Finally, doses of dermatan sulfate which achieved the same antithrombotic effect as heparin, did not enhance bleeding.

The objective of this thesis is to explore these observations further, to determine if the catalysis of thrombin inhibition by heparin cofactor II can as effectively, or more effectively achieve an antithrombotic effect, than the
catalysis of thrombin inhibition by antithrombin III. This thesis will discuss, first, the current dogma concerning the activation and regulation of the coagulation system, the mechanisms of action of heparin and dermanan sulfate, and the current rationale for their use as antithrombotic agents. Then, the clinical and experimental evidence which supports this rationale will be presented and unresolved issues associated with the use of both heparin and dermanan sulfate will be identified. It is on the basis of these unresolved issues that a number of questions have been raised, and that a number of experiments have been performed to address these questions. These studies focus predominantly on the relative benefits and the related mechanisms of action of dermanan sulfate and heparin i) in the prevention of thrombus formation (prophylaxis); ii) in the prevention of thrombus growth (treatment); iii) when used as adjuncts to fibrinolytic therapy; and iv) in the prevention of arterial thrombosis.
I. COAGULATION AND THROMBUS FORMATION

Hemostasis is the normal process by which bleeding is prevented following blood vessel injury. This involves coordinated interactions among the blood vessel wall, circulating platelets, circulating coagulation proteins and endogenous fibrinolytic agents (Colman et al, 1987). Under normal physiological conditions, the interactions among these different components are delicately balanced such that blood flows freely until an injury triggers hemostasis. The coagulation system plays a crucial role in hemostasis, since dysfunctional coagulation factors or their inhibitors can lead either to prolonged bleeding or to thrombosis (Colman et al, 1987).

The coagulation system consists of a series of enzymatic reactions, where the product of each reaction acts as the enzyme in the subsequent reaction, resulting in a "cascading effect" (Figure 1, MacFarlane, 1964; Davie and Ratnoff, 1964). There are two pathways involved in coagulation, the intrinsic and extrinsic pathways, which are closely linked through a number of reactions (Figure 1).

The intrinsic pathway is initiated when the zymogen factor XII is converted into the enzyme factor XIIa (Davie and Fujikawa, 1975). This is thought to occur when blood comes into contact with a de-endothelialized vessel wall or a foreign surface. Factor XIIa then converts factor XI to the enzyme factor XIa (Ratnoff et al, 1961). Factor XIa activates
Figure 1: A summary of the different pathways in the coagulation cascade and various feedback mechanisms (dashed lines).
factor IX in a reaction which is calcium dependent (Fujikawa et al., 1974). Factor IXa, phospholipid and calcium then activate factor X into factor Xa in the presence of factor VIIIa (Lundblad and Davie, 1964; Hemker and Kahn, 1967). Factor VIII is activated by the action of trace amounts of factor Xa and/or thrombin (Rapaport et al., 1963; Foster et al., 1983; Ofosu et al., 1987a; Ofosu et al., 1989).

Factor X is also activated by the extrinsic pathway. The extrinsic pathway is initiated when the vascular endothelium and/or extravascular tissues are damaged and tissue thromboplastin is exposed to the circulating blood (Davie and Fujikawa, 1975). Tissue thromboplastin forms a complex with factor VII, which in the presence of calcium, activates factor X (Williams and Norris, 1966). Thus, activation of factor X is a common step in the extrinsic and intrinsic pathways (Figure 1). Activated factor X, in the presence of calcium, phospholipid and activated factor V, converts prothrombin into thrombin (Bull et al., 1972; Davie and Fujikawa, 1975). These reactions are localised on the phospholipid surface of various activated cells, including platelets, macrophages and injured endothelial cells (Rogers and Shuman, 1983; Rosing et al., 1985; Tracy et al., 1985). Once thrombin is formed, it cleaves fibrinogen resulting in the formation of fibrin monomers which subsequently polymerize (Doolittle, 1981; Marder et al., 1982). These fibrin polymers are stabilized by factor XIIIa. Factor XIII is activated by
thrombin, in a calcium-dependent reaction (Chung et al, 1974).

The extrinsic and intrinsic pathways are also connected by a number of feedback mechanisms. To enhance coagulation further, both factor Xa and thrombin activate factor VIII and factor V (Ofosu et al, 1987a). Factor VII is also activated by factors IXa, XIIa and XIa. Factor VIIa complexed with tissue factor also activates factor IX (Zur and Nemerson, 1980).

A number of endogenous inhibitors of coagulation participate in regulating thrombin formation and activity. Antithrombin III, a glycoprotein with a molecular weight of 58,000 daltons, is present in the plasma in concentrations of 150 μg/ml (Abildgaard, 1968; Rosenberg, 1989). Antithrombin III is the principle endogenous inhibitor of not only thrombin, but also factors Xa and IXa (Barrowcliffe and Thomas, 1987). It should be noted that a number of other thrombin inhibitors have also been identified. These include heparin cofactor II, which binds to thrombin at the same active site as antithrombin III and inactivates thrombin (Briginshaw and Shanberge, 1974; Tollefsen et al, 1982), α₂-macroglobulin and α₁-antitrypsin. The latter two inhibitors are thought to be more important in regulating fetal coagulation (Andrew et al, 1987). The relative importance of antithrombin III and heparin cofactor II will be discussed in more detail in their relationship to their interactions with heparin and dermatan sulfate. Another regulator of thrombin
activity is the thrombomodulin/protein C pathway (Esmon, 1983; Esmon and Esmon, 1984; Clouse and Comp, 1986). Thrombomodulin is present on the endothelium and binds thrombin. This complex prevents the thrombin-induced activation of factors V and VIII and limits the thrombin-induced conversion of fibrinogen into fibrin (Esmon et al, 1982). The thrombin-thrombomodulin complex also activates protein C, which in turn inactivates factors Va and VIIIa thereby limiting prothrombinase formation (Esmon, 1983; Clouse and Comp, 1986). The role of the thrombomodulin/protein C pathway in thrombin regulation was not investigated in this thesis.

In summary, coagulation is regulated by a number of enzymatic reactions, some of which facilitate coagulation and others which inhibit coagulation. This delicate balance must be maintained to prevent thrombosis. However, if a stimulus which initiates coagulation is greater than the regulatory effect of endogenous coagulation inhibitors, thrombosis can result. Thus the rationale for the use of heparin in the prevention and treatment of thrombosis is to reverse this imbalance in coagulation, by catalysing the inhibition of procoagulant enzymes by the endogenous inhibitor, antithrombin III.
II. ANTICOAGULANT PROPERTIES OF HEPARIN AND DERMATAN SULFATE

1. Structure and heterogeneity of heparin and dermatan sulfate

Heparin and dermatan sulfate are glycosaminoglycans which consist of repeating disaccharide residues. Heparin is synthesized and stored in mast cells found in many mammalian tissues including the lung, liver, skin and intestinal mucosa (Jaques, 1980; Björk and Lindahl, 1982; Hassell et al, 1986). Dermatan sulfate is synthesized by a variety of cells, including epithelial cells, and vascular smooth muscle and endothelial cells. Dermatan sulfate is normally found in skin, heart valves, tendons and arterial blood vessels (Lindahl and Höök, 1978; Hassell et al, 1986). Dermatan sulfate has been isolated from the intima of the arterial vessel wall, where it is thought to influence the structure and permeability of the vessel wall and to interact with flowing blood (Lindahl and Höök, 1978; Hassell et al, 1986).

Both heparin and dermatan sulfate have been isolated as either free polysaccharide chains or as proteoglycans (Jaques, 1980; Björk and Lindahl, 1982). Proteoglycans contain one or more glycosaminoglycan chains which are covalently bound to a protein core (Hassell et al, 1986). There is, however, a large degree of diversity in these glycosaminoglycan chains, both in their length and chemical composition. These differences can account for their diverse range of functions (Lindahl and Höök, 1978; Casu, 1985).
The major repeating disaccharides of heparin are D-glucosamine and L-iduronic acid (Figure 2), which comprise ~60-70% of the heparin chains. Heparin chains contain 1.6 - 3.0 sulfate groups/disaccharide (Casu, 1985). The molecular weight of these heparin chains ranges between 6,000 - 25,000 daltons (mean \( M_r = 15,000 \), Casu, 1985). The major repeating disaccharides of dermatan sulfate are D-galactosamine and L-iduronic acid (Figure 2, Lindahl and Höök, 1978). Dermatan sulfate contains 1.5 - 2.0 sulfate groups/disaccharide (Casu, 1985). The molecular weight of the dermatan sulfate chains ranges between 15,000 - 40,000 daltons (mean \( M_r = 20,000 \), Casu et al, 1985).

Both heparin and dermatan sulfate require a minimum amount of sulfation for their anticoagulant activities. Nagasawa et al (1977) N-desulfated heparin and found that this procedure decreased its anticoagulant activity. When the same heparin was N-resulfated, the anticoagulant activity was restored to 94% of its original value. Similar desulfation studies have not been performed with dermatan sulfate. Other studies, however, have shown that in vitro sulfation of dermatan sulfate increases its anticoagulant activity (Ofosu et al, 1987b; Dol et al, 1988a; Fischer et al, 1989). These results suggest that sulfation of both heparin and dermatan sulfate is required for anticoagulant activity.
Figure 2: The major repeating disaccharides of heparin and dermatan sulfate. Taken from Casu, (1985).
2. Anticoagulant actions of heparin

(a) Catalysis of thrombin and factor Xa inhibition by antithrombin III

Antithrombin III is a circulating endogenous inhibitor of factors IXa, Xa and thrombin (Damus et al, 1977; Colman et al, 1989). Antithrombin III neutralizes these procoagulant enzymes by binding covalently to their active serine site, thereby forming a stable antithrombin III-procoagulant enzyme complex (Barrowcliffe and Thomas, 1987). Heparin catalyses the inhibition of thrombin, factors Xa and IXa by antithrombin III in plasma at therapeutic concentrations (Scott et al, 1982; McNeely and Griffith, 1985; Pixley et al, 1985; Colman et al, 1989). To achieve this effect, a specific pentasaccharide sequence of heparin must bind to antithrombin III (Figure 3A, Choay et al, 1983; Lindahl et al, 1983). Fragments of heparin which contain this pentasaccharide sequence and are less than 18 monosaccharides in length (\(M_r \leq 4500\)) bind to antithrombin III, but do not catalyse thrombin inhibition (Danielsson et al, 1986). These fragments however, can effectively catalyse factor Xa inhibition (Choay et al, 1983; Atha et al, 1987). This suggests that heparin chains must be sufficiently long to bind both thrombin and antithrombin III in order to catalyse thrombin inhibition (Figure 3B, Barrowcliffe and Thomas, 1987). Heparin binds to thrombin at the anion binding exosite(s) of thrombin, which are positively charged regions which become exposed when
Figure 3: Diagrammatic representation of heparin, antithrombin III, factor Xa and thrombin (A) and of heparin-induced catalysis of thrombin (B) and factor Xa (C) inhibition by antithrombin III.
thrombin is generated (Fenton et al, 1988; Church et al, 1989; Fenton, 1989). The catalysis of factor Xa inhibition however, only requires the presence of the antithrombin III-binding sequence of heparin (Figure 3C). This explains why low molecular weight heparin fractions (M₆ ~5000) have a higher anti-factor Xa:anti-thrombin ratio than unfractionated heparin (Ofosu and Barrowcliffe, 1990).

(b) Catalysis of thrombin inhibition by heparin cofactor II

Heparin cofactor II is also a circulating endogenous inhibitor of thrombin (plasma concentration 90 μg/ml). It does not inhibit other coagulation proteases or fibrinolytic components (Ofosu et al, 1984a, Parker and Tollefsen, 1985). Heparin catalyses the rate of thrombin inhibition by heparin cofactor II ~1000 fold in purified systems (Tollefsen et al, 1983). However, the concentration of heparin which is required to catalyse thrombin inhibition by heparin cofactor II is much higher than the concentration required to catalyse thrombin inhibition by antithrombin III, i.e. heparin plasma concentrations which are not normally achieved clinically (Tollefsen and Blank, 1981; Abildgaard and Larsen, 1984; Ofosu et al, 1985; Scully et al, 1987). These observations have contributed to the conclusion that the catalytic effect of heparin on heparin cofactor II is of lesser importance than the catalytic effect of heparin on antithrombin III.

However, other observations provide evidence that
heparin cofactor II may be more biologically important than first thought (Ofosu et al, 1985). When Ablidgaard and Larsen (1984) investigated the ability of heparin to catalyse thrombin inhibition by heparin cofactor II in vitro, they did so in diluted human plasma. When Ofosu et al (1985) investigated the ability of heparin to catalyse thrombin inhibition by heparin cofactor II, they did so in undiluted plasma and found that heparin (at therapeutic concentrations) significantly catalysed thrombin inhibition by heparin cofactor II. Furthermore, the heparin-induced catalysis of thrombin inhibition by heparin cofactor II was also enhanced in the presence of calcium and tissue factor (Ofosu et al, 1985), while the heparin-induced catalysis of thrombin inhibition by antithrombin III was significantly reduced under the same conditions (Ofosu et al, 1984b, Barrowcliffe et al, 1987). [As indicated in Figure 1, many interactions in the coagulation cascade including both acceleration and inhibition reactions are calcium- and/or phospholipid-dependent.] Thus, heparin also effectively catalysed the inhibition of thrombin by heparin cofactor II when tested using experimental conditions which better mimic in vivo physiological conditions.

(c) **Heparin cofactor independent effects**

Heparin has also been shown to prolong the thrombin clotting time and to delay prothrombin activation in plasma depleted of heparin cofactor II and antithrombin III (Sie et
al, 1986; Ofosu et al, 1986). The latter effect appears to be achieved either by inhibiting prothrombinase complex formation or by preventing activation of prothrombin (Ofosu et al, 1986, 1987a). However, the significance of these observations in vivo are not known.

3. Anticoagulant action of dermatan sulfate

(a) Catalysis of thrombin inhibition by heparin cofactor II

The rate of thrombin inhibition by heparin cofactor II in vitro is also increased ~1300-fold by optimal dermatan sulfate concentrations in a purified system (Tollefsen et al, 1983). Dermatan sulfate consists of fractions which bind heparin cofactor II with either high affinity or low affinity (Tollefsen et al, 1986). Griffith and Marbet (1983) and Tollefsen et al (1986) found that the fractions of dermatan sulfate which bind heparin cofactor II with high affinity were more negatively charged. Consistent with these observations, Maimone and Tollefsen (1990) recently isolated a highly sulfated hexasaccharide of dermatan sulfate which may constitute the binding site for heparin cofactor II, since this hexasaccharide binds heparin cofactor II with high affinity.

The length of the dermatan sulfate chains required for thrombin inhibition has also been studied (Maimone and Tollefsen, 1990; Tollefsen et al, 1986). Fragments of dermatan sulfate which are less than 6 monosaccharides do not
bind heparin cofactor II. Fragments of dermatan sulfate which contain 6 to 13 monosaccharides bind heparin cofactor II but do not catalyse thrombin inhibition. Fragments which contain ≥14 monosaccharides, however, bind to heparin cofactor II and catalyse thrombin inhibition (Tollefsen et al, 1986). These observations suggest that dermatan sulfate, like heparin, must be a certain length in order to achieve its catalytic effect (Figure 4).

4. Relative importance of factor Xa and thrombin inhibition

Unfractionated heparin, by convention, has an equal ability to inhibit both factor Xa and thrombin. However, the relative importance of these two activities in achieving an anticoagulant effect has received considerable attention. Over the last decade it has been suggested that the catalysis of factor Xa inhibition results in a better antithrombotic effect than the catalysis of thrombin inhibition (Carter et al, 1982, Mattson et al, 1985; Walenga et al, 1987). This is based upon the observations that low molecular weight heparin fractions, which have higher anti-factor Xa activity than anti-thrombin activity, were as effective as heparin in preventing thrombus formation (Cade et al, 1984). However, Ofosu et al (1985) demonstrated that a heparin fragment (an octasaccharide) which had only anti-factor Xa activity was a poor inhibitor of factor Xa-induced thrombin generation. Thus when the octasaccharide fragment was added to plasma, there
Figure 4: Diagrammatic representation of dermatan sulfate, heparin cofactor II and thrombin (A), and of dermatan sulfate-induced catalysis of thrombin inhibition by heparin cofactor II (B).
was a concentration-dependent inhibition of factor Xa, but no inhibition of thrombin generation. Interestingly, dermatan sulfate had the opposite effect. When dermatan sulfate was added to plasma, there was a concentration-dependent inhibition of thrombin generation, and no inhibition of factor Xa.

These latter observations suggest that it is more important to inhibit thrombin generation to achieve an antithrombotic effect, since the selective inhibition of factor Xa by heparin-like compounds does not prevent thrombin generation, and thus, fibrin formation.

III. PHARMACOKINETICS AND PHARMACODYNAMICS: POTENTIAL INFLUENCE ON COAGULATION

1. Intravenous heparin

Heparin appears to be cleared from the circulation by both a saturable and non-saturable mechanism, based on studies performed both in animals and in man (Dawes and Pepper, 1979; Jaques, 1980; de Swart et al, 1982; Boneu et al, 1987a). When heparin was injected into rabbits in doses of 1-100 U/kg, heparin clearance was exponential and followed first-order kinetics, where the half-life of heparin increased as the dose of heparin was increased. When doses of heparin which exceeded 100 U/kg were injected, the half-life of heparin remained constant as the dose of heparin was increased. Heparin clearance at these higher doses followed
a concave-convex pattern, suggesting that heparin was cleared by two routes, by a saturable and non-saturable route. Similarly, the disappearance of the anticoagulant activity of heparin, measured as either anti-factor Xa activity or a prolongation in the activated partial thromboplastin time (APTT), followed a similar pattern both in human volunteers and in rabbits (Dawes and Pepper, 1979; de Swart et al, 1982; Boneu et al, 1987a). These observations suggest that at lower doses, heparin is cleared predominantly by a saturable mechanism and that at higher doses, the former mechanism becomes saturated, and heparin is cleared predominantly by a non-saturable mechanism.

The saturable mechanism of heparin clearance is thought to involve both the vascular endothelium and the reticuloendothelial system (Estes, 1971; Dawes and Pepper, 1979; de Swart, 1982). Heparin binds to rat endothelium in vivo (Hiebert and Jaques, 1976; Mahadoo et al, 1977) and to cultured endothelial cells in vitro (Barzu et al, 1984). In the latter study, most of the heparin was internalized by the endothelial cell within 4-5 hours. Once inside the cells, the heparin was reported to be degraded by lysosomal enzymes, since the heparin recovered from the endothelial cells had a reduced molecular weight (Barzu et al, 1985; 1987).

The non-saturable mechanism of heparin clearance is thought to involve the renal system. Caranobe et al (1985) found that the clearance of low dose heparin was similar in
control and nephrectomized rabbits, but the clearance of high
dose heparin (>100 U/kg) was significantly impaired in
nephrectomized rabbits. Consistent with these observations,
Dawes and Pepper (1979) found that when a low dose of $^{131}$I-
heparin was administered to human volunteers, all of the $^{131}$I-
heparin was recovered in the urine as degraded heparin ($M_r \leq$
1000). This is consistent with the hypothesis that the
injected $^{131}$I-heparin had been internalized by the endothelial
cells and subsequently metabolized. When a high dose of $^{131}$I-
heparin was administered to the same volunteers, the majority
of the $^{131}$I-heparin was recovered in the urine as intact
heparin i.e. with the same molecular weight as the starting
material. These observations are consistent with the
possibility that most of the high dose heparin was cleared
intact by the renal system.

The doses of heparin used to prevent venous thrombosis
clinically are cleared predominantly by the saturable or
cellular mechanism (Boneu et al, 1987a; de Swart et al, 1982).
However, the higher doses of heparin used to treat venous
thrombosis (when given as a continuous infusion) appear to
saturate the cellular mechanism within 24 to 48 hours in most
patients and are then also cleared by the non-saturable
mechanism (de Swart et al, 1982; Prins and Hirsh, 1991). High
bolus doses of heparin, which are cleared predominantly by the
non-saturable mechanism, are usually only administered in
select situations, such as during cardiopulmonary bypass in
cardiac surgery (Spencer, 1983).

2. Intravenous low molecular weight heparin fractions

The clearance of low molecular weight heparin fractions differs significantly from that of unfractionated heparin. Low molecular weight heparin fractions are cleared more slowly than heparin when given in doses routinely used clinically. Interestingly, the anti-factor Xa activity of the low molecular weight heparin fractions is cleared more slowly than their anti-thrombin activity (Briant et al, 1989). Boneu and colleagues (Caranobe et al, 1985; Boneu et al, 1987b) also demonstrated that the clearance of both low and high doses of low molecular weight heparin was significantly impaired in nephrectomized rabbits. These observations are consistent with the in vitro observations that low molecular weight heparin fractions do not bind as effectively to the endothelium as heparin, and suggest that low molecular weight fractions are cleared mostly by the non-saturable renal mechanism.

The above observations provided the basis for the rationale that low molecular weight heparin fractions may be more useful than unfractionated heparin as antithrombotic agents, since low molecular weight heparin fractions were cleared more slowly. Therefore, low molecular weight heparins could be administered less frequently than heparin to achieve the same anticoagulant activity.
3. Intravenous dermatan sulfate

Clearance of dermatan sulfate also appears to depend upon renal function (Dol et al, 1988b), since dermatan sulfate clearance is independent of the dose administered, and follows a pattern of clearance similar to low molecular weight heparins when tested in rabbits.

4. Importance of glycosaminoglycan clearance for achieving anticoagulant and antithrombotic effects

In summary, heparin is cleared from plasma by a saturable, cellular mechanism and a non-saturable renal mechanism. However, low molecular weight heparin fractions and dermatan sulfate are cleared mainly by the non-saturable renal mechanism.

The differences in clearance of these compounds may be important for their relative anticoagulant and antithrombotic effects. For example, binding of heparin to the endothelium may be beneficial, since heparin bound to the vessel wall near a site of injury may suppress subsequent thrombus formation. Alternatively, binding of heparin to the vessel wall may be detrimental, since the circulating anticoagulant activity may be lost more rapidly than that of low molecular weight heparins and dermatan sulfate. As a result, the antithrombotic potential of heparin may decrease. Subsequent studies in this thesis will address these two issues from the perspective of the differences in the abilities of heparin and dermatan sulfate to catalyse the
inhibition of circulating thrombin versus thrombin bound to a thrombus, and how these differences may influence their antithrombotic effects.

IV. THE PREVENTION OF VENOUS THROMBOSIS BY HEPARIN AND DERMATAN SULFATE

1. Clinical use of heparin in the prevention of venous thrombosis

Venous thrombosis and pulmonary embolism are important complications in hospitalized patients. In addition, a number of people in the general population develop idiopathic venous thrombosis as well as pulmonary embolism. The major risk factors associated with these thromboembolic complications are venous stasis, trauma and age (Havig, 1977; Carter and Gent, 1982; Coom, 1984; Janssen et al, 1987). Venous stasis can be caused by immobility, congestive heart failure, and venous obstruction particularly in patients over 50 years of age. Trauma-induced venous thrombosis is usually associated with surgery.

Venous thrombi usually form in regions of disturbed or impaired flow, such as in valve cusp pockets or venous sinuses in the leg (Gallus and Hirsh, 1976). These thrombi are non-organized fibrin/red cell masses with a small platelet aggregate head. As the thrombi propagate, mainly in the direction of blood flow, they partially or completely occlude the vessel lumen. If the thrombi are not firmly attached to
the vessel wall at their site of origin, they may embolise and locate elsewhere in the blood vasculature, such as in the pulmonary bed.

Heparin has been used to prevent and to treat venous thrombosis for 35-40 years (Barrit and Jordan, 1960; Gallus and Hirsh, 1976). Low dose heparin has been shown to effectively prevent deep vein thrombosis in general medical and surgical patients (Kakkar et al, 1972; Gallus et al, 1973; International Multicentre Trial, 1975; Kiil et al, 1978; Colditz et al, 1986). There are, however, a select group of patients who are not protected by low dose heparin and require a higher dose of heparin to prevent thrombosis (Mannucci et al, 1976; Leyvraz et al, 1983). This group of patients includes those undergoing major orthopedic surgery, such as hip or knee replacement surgery or fractures of the femoral neck (Hampson et al, 1974; Hull and Hirsh, 1979; Hyers et al, 1986).

The explanation for this difference in heparin requirement is not entirely clear. One possibility is that the stimulus which initiates thrombus formation is different in the different patient groups. For example, orthopedic surgery may result in more vascular and tissue damage, resulting in the generation of tissue factor and widespread hypercoagulation, while a more localised activation of coagulation may occur in patients undergoing general surgery, or in patients with idiopathic deep vein thrombosis. Thrombus
formation in the latter cases may be initiated by activated factor X or thrombin at the injury site. It is possible that the abilities of heparin and dermatan sulfate to prevent thrombus formation initiated by these various procoagulant stimuli may differ. This issue will be addressed in more detail in this thesis.

Low molecular weight heparins are being used with increased frequency as an alternative to low dose heparin prophylaxis. The rationale for this change in therapy is that low molecular weight heparin fractions can be administered as a single subcutaneous dose each day and can be as effective as heparin, which must be administered as 2 to 3 subcutaneous doses each day (Kakkar and Murray, 1985; Caen, 1988). This is based upon the observations that low molecular weight heparin fractions are cleared more slowly than unfractionated heparin, and therefore their anticoagulant effects persist for a longer period of time in plasma. It should be noted, however, that the doses of low molecular weight heparin fractions used in these clinical studies generated circulating anti-factor Xa levels of 0.1 to 0.2 U/ml. These levels were 2-fold higher than the anti-factor Xa levels generated with low dose heparin (Caen, 1988). However, the anti-thrombin levels generated in the patients receiving low molecular weight heparin fractions were the same as the anti-thrombin levels generated in the patients receiving heparin. The relevance of these two points will be discussed later.
2. Heparin and dermatan sulfate in the prevention of venous thrombosis in experimental models

(a) Stimulus initiating thrombosis

Over 100 years ago, Virchow (1884) demonstrated that a combination of stasis, hypercoagulation and vessel wall injury would result in venous thrombus formation. However, the relative importance of each parameter for thrombus formation is not clearly understood. Hypercoagulation, stasis or vessel wall damage alone does not result in thrombus formation (Wessler et al, 1959; Aronson and Thomas, 1985).

It has also been demonstrated that the ability of heparin to prevent thrombus formation depends in part on which stimulus initiates the hypercoagulable state. Gite1 et al (1977) injected animals with either factor IXa, factor Xa or thrombin and then induced stasis in the jugular vein to produce a thrombus. They found that the dose of heparin required to inhibit thrombus formation differed depending on the procoagulant used. These observations demonstrate that the ability of heparin to catalyse the inhibition of specific enzymes in the coagulation cascade differs in vivo. This may, in part, provide an explanation for the difference in heparin requirements needed to prevent venous thrombosis in patients undergoing general surgery versus orthopedic surgery.

Since dermatan sulfate only catalyses thrombin inhibition and not other components of the coagulation cascade, it is possible that dermatan sulfate may be more
effective than heparin in certain prothrombotic conditions.

(b) Relative importance of anti-factor Xa activity and anti-thrombin activity

The relative importance of inhibiting factor Xa versus inhibiting thrombin to achieve an anticoagulant effect has major implications for the relative abilities of heparin and related compounds to prevent thrombus formation. Several studies have investigated the importance of catalysing factor Xa or thrombin inhibition in the prevention of thrombus formation. Walenga et al (1987) compared the abilities of a pentasaccharide heparin (the sequence required for antithrombin III binding which only catalyses the inhibition of factor Xa) and heparin to prevent thrombus formation in vivo. They demonstrated that ~80 anti-factor Xa U/kg of the pentasaccharide inhibited thrombus formation, compared to ~15 anti-factor Xa and anti-thrombin U/kg of heparin. Thus, it was concluded that the pentasaccharide effectively inhibited thrombus formation by catalysing the inhibition of factor Xa, albeit at higher doses than those required by heparin.

Buchanan et al (1985) compared the abilities of heparin, an octasaccharide heparin fragment (which also contained the pentasaccharide sequence and had only anti-factor Xa activity) and also dermatan sulfate (which only catalyses the inhibition of thrombin by heparin cofactor II) to prevent thrombus formation in vivo. They found that heparin inhibited thrombus formation by 90% when given in a
dose of 10 anti-factor Xa and anti-thrombin U/kg. When the octasaccharide was given in a comparable dose (equivalent anti-factor Xa activity), it only inhibited thrombus formation by 41%. In contrast, dermatan sulfate inhibited thrombus formation by 90% when given in a dose of 3 anti-thrombin U/kg. Thus, it was concluded that the catalysis of factor Xa inhibition by the octasaccharide did not effectively inhibit thrombus formation, while the catalysis of thrombin inhibition by dermatan sulfate inhibited thrombus formation as effectively as heparin.

The conclusions of these two studies appear to be contradictory, however, it has subsequently been demonstrated that the antithrombotic effect achieved by high doses of the pentasaccharide may not be due to the inhibition of factor Xa alone. Ofosu et al. (1990) demonstrated that the pentasaccharide also inhibited thrombin by heparin cofactor II at plasma concentrations (i.e. 50-fold higher than heparin on a molar basis) achieved by the antithrombotic dose used in the study by Walenga et al. (1987). Therefore, both studies demonstrate the importance of catalysing thrombin inhibition to achieve an antithrombotic effect.

These in vivo studies and the studies performed by Ofosu et al. (1985) in vitro (see page 18) demonstrated that i) the catalysis of factor Xa inhibition alone was not sufficient to achieve an antithrombotic effect; ii) that glycosaminoglycans with only anti-thrombin activity were
effective antithrombotic agents, even when factor Xa activity was not inhibited; iii) dermatan sulfate was a potent antithrombotic agent; and iv) a significant antithrombotic effect could be achieved by catalysing thrombin inhibition by heparin cofactor II.

As a consequence, further studies were performed to investigate the relationship between the antithrombotic effects of dermatan sulfate and heparin, and their anticoagulant effects. It was confirmed that dermatan sulfate was effective in preventing thrombus formation in stasis/hypercoagulation models (Fernandez et al, 1986a, Merton and Thomas, 1987; Maggi et al, 1987; Dol et al, 1990). The antithrombotic effects of dermatan sulfate in these studies were achieved with doses which did not prolong the TCT or the APTT and which did not generate any anti-factor Xa activity. In addition, it was confirmed that the antithrombotic effect of dermatan sulfate was associated with the catalysis of thrombin inhibition by heparin cofactor II.

V. THE TREATMENT OF VENOUS THROMBOSIS BY HEPARIN AND DERMATAN SULFATE

Higher doses of heparin are required to prevent thrombus growth or extension than are required to prevent thrombus formation (Gallus and Hirsh, 1976; Walker et al, 1987; Ofcsu et al, 1990). The explanation for this difference is not clear. It has been postulated that a higher heparin
requirement probably reflects the cascading characteristic of the coagulation pathway, and that more heparin is required to inhibit thrombin once it is formed, than is required to prevent its initial generation (Ofosu et al, 1987a). Alternatively, it is possible that thrombin which is bound to a thrombus is more resistant to inhibition by heparin than free thrombin which is circulating in plasma.

In support of this possibility, Hogg and Jackson (1989) found that the ability of heparin to catalyse the inhibition of thrombin by antithrombin III was reduced 300-fold when thrombin was bound to fibrin monomers as compared to when thrombin was free in solution. Weitz et al (1990) also demonstrated that a 20-fold higher concentration of heparin was required to inhibit thrombin bound to fibrin to a similar degree as was required to inhibit thrombin which was free in solution. These results indicate that thrombin bound to a fibrin-rich thrombus may also be protected from inhibition by heparin/antithrombin III. This is illustrated diagrammatically in Figure 5. Similarly, Bar-Shavit et al (1989) found that thrombin bound to subendothelial extracellular matrix components was also resistant to inhibition by heparin.

These observations provided a possible explanation for results obtained in an earlier study by Boneu et al (1985). It was demonstrated in rabbits, that a dose of heparin which was comparable to that used clinically, inhibited the
Figure 5: Diagrammatic representation of thrombin bound to fibrin (A) and its protection from inhibition by heparin/antithrombin III (B).
accretion of radiolabelled fibrin onto existing thrombi by 50% and increasing the dose 7-fold had no further effect. Similar results were obtained with a number of low molecular weight heparin fractions, which also inhibited fibrin accretion by 50%. In contrast, when animals were treated with a heparinoid (which contained 15% dermatan sulfate), fibrin accretion was inhibited by 75%. Furthermore the antithrombotic effect of the heparinoid was associated with the catalysis of thrombin inhibition by heparin cofactor II as well as antithrombin III (Boneu et al, 1985; Fernandez et al, 1987). These investigators raised the possibility that the dermatan sulfate component of the heparinoid contributed significantly to its antithrombotic effect. The observations that thrombin is resistant to inhibition by heparin/antithrombin III when thrombin is bound to a surface, provides a possible explanation for the reduced effectiveness of heparin and why a higher dose is required to prevent fibrin accretion onto an existing thrombus. The observation that the heparinoid inhibited fibrin accretion more effectively than heparin, suggests that thrombin bound to fibrin is more susceptible to inhibition by dermatan sulfate/heparin cofactor II. This will be tested in this thesis.

VI. HEPARIN INTERACTIONS WITH tPA ON FIBRINOLYSIS

1. Fibrinolysis and the mechanism of action of tPA

It has been suggested that fibrinolysis may be
enhanced in the presence of heparin since the prevention of thrombus growth would facilitate endogenous fibrinolysis (Loscalzo and Braunwald, 1988). Endogenous degradation of fibrin occurs by plasmin, an enzyme produced by the activation of its precursor, plasminogen. Plasminogen is normally present in the circulation and can be activated by several endogenous plasminogen activators (Verstraete and Collen, 1986). One endogenous plasminogen activator is tissue plasminogen activator (tPA) which is synthesized by endothelial cells, and is released when the endothelial cells are activated by a number of stimuli, including thrombin (van Hinsberg, 1988).

TPA is a serine protease ($M_r = 70,000$), with a half-life in plasma of about 4 minutes in man (Loscalzo and Braunwald, 1988; van Hinsberg, 1988, Collen et al, 1989). TPA has a high affinity for fibrin and once bound, activates fibrin-bound plasminogen. In the absence of fibrin, tPA is a poor activator of plasminogen, however, in the presence of fibrin, plasminogen activation can be enhanced 600-fold (Verstraete and Collen, 1986; Loscalzo and Braunwald, 1988). Activated plasminogen, i.e. plasmin, which is bound to fibrin is protected from inactivation by the endogenous plasmin inhibitor, $\alpha_2$-antiplasmin (Verstraete and Collen, 1986; Collen et al, 1989). In contrast, any circulating plasmin is rapidly inactivated by $\alpha_2$-antiplasmin. As fibrin is degraded, new tPA binding sites become exposed and further plasminogen
activation and fibrin degradation occur, thereby allowing for the dissolution of an existing thrombus (Verstraete and Collen, 1986).

2. *Interactions between tPA and heparin*

(a) **Clinical studies**

*tpA has been used to lyse platelet/fibrin thrombi, thereby restoring coronary artery patency in patients after myocardial infarction. It has also been used to treat deep vein thrombosis and pulmonary embolism* (Verstraete and Collen, 1986; Loscalzo and Braunwald, 1988; Collen et al, 1989). Heparin is also usually administered concurrently with tPA in thrombolytic therapy (Collen et al, 1989; Topol et al, 1989). The rationale for using the combination of tPA plus heparin, is based upon the concept that tPA-induced fibrinolysis will be enhanced if thrombus growth is prevented by heparin.

Recent clinical studies have demonstrated that patency in infarct-related coronary arteries was maintained for up to one week following thrombolysis in a greater number of patients receiving tPA and heparin, than in patients receiving tPA alone (Prins and Hirsh, 1991; Hsia et al, 1990; Bleich et al, 1990). However it was also demonstrated that the simultaneous administration of heparin and tPA did not reduce the incidence of reinfarction, recurrent ischemia or mortality compared to the administration of tPA alone (GISSI-2, 1990; Topol et al, 1989; Bleich et al, 1990). Thus, the co-administration of high dose heparin is currently recommended
with tPA in the treatment of myocardial infarction (Prins and Hirsh, 1991). However, the need for high dose heparin in this clinical situation suggests that tPA and heparin do not act synergistically and/or that the thrombus remains thrombogenic during thrombolytic therapy. Experimental studies discussed below provide further insight into these clinical observations.

(b) Experimental studies

A number of investigators have demonstrated that tPA is an effective thrombolytic agent without causing any significant fibrinogenolysis in experimental models (Bergmann et al., 1983; Agnelli et al., 1985a; 1985b). In these studies, tPA was given alone, not in combination with heparin. To investigate the possible interactions between tPA and heparin, Andrade-Gordon and Strickland (1986) demonstrated that the ability of tPA to activate plasminogen in the presence of fibrin was impaired when heparin was added to a purified system in vitro. Paques et al. (1986) performed similar in vitro studies and confirmed the above results, and concluded that heparin and fibrin compete for tPA binding. Fry and Sobel (1988) measured tPA-induced lysis of thrombi in the presence or absence of heparin in vitro, and found no difference in tPA-induced fibrinolysis. However, Cercek et al. (1986) found that a combination of tPA and heparin was more effective than tPA alone, in reducing thrombus size in a canine arterial thrombus model (Cercek et al., 1986). This
latter study suggests that there was synergism between tPA and heparin. Agnelli et al (1990) measured tPA-induced lysis of venous thrombi in rabbits in the presence and absence of heparin and found no difference in tPA-induced thrombolysis. However, this study also examined the effects of a co-administration of tPA and heparin on the ability of heparin to prevent thrombus growth. It was demonstrated that a dose of heparin which prevented thrombus growth in the absence of tPA was ineffective in preventing thrombus growth in the presence of tPA. This suggests that heparin is not as effective in preventing thrombus growth during thrombolytic therapy and may explain why larger doses of heparin are required in the clinical setting.

The interactions between tPA, fibrin and dermatan sulfate have not been tested. However, Andrade-Gordon and Strickland (1986) found that dermatan sulfate did not interact with tPA and plasminogen to the same extent as heparin did. These observations suggest that dermatan sulfate does not interfere with tPA-induced fibrin degradation.

To further investigate the interactions between [tPA and heparin] or [tPA and dermatan sulfate] on tPA-induced thrombolysis, different doses of either heparin or dermatan sulfate will be co-administered with a dose of tPA which results in an ~50% lysis. This will allow the determination of any synergistic or antagonistic effects between these compounds in vivo.
VII. HEMORRHAGIC SIDE-EFFECTS

Heparin is used successfully for the prevention and treatment of a number of thrombotic events. However, these beneficial effects of heparin can be associated with increased bleeding. Many attempts have been made to identify which property (or properties) of heparin contribute to its bleeding side-effect. There is no consistent relationship between the anti-factor Xa and anti-thrombin activities of various heparin preparations or other glycosaminoglycans and their abilities to enhance blood loss (Buchanan et al, 1986; Ockelford et al, 1982; Andriuoli et al, 1985; Fernandez et al, 1986b).

One property of these glycosaminoglycans which is associated with their ability to enhance blood loss, however, is their extent of sulfation. Thus, it was demonstrated for a number of glycosaminoglycans, that the greater the degree of sulfation, the greater the ability to enhance blood loss (Van Ryn-McKenna et al, 1989). It is possible that the enhanced negative charge associated with highly sulfated glycosaminoglycans influences their ability to bind to surfaces in vivo, such as to platelet membranes or to the vessel wall. Consistent with this possibility, a previous study showed that glycosaminoglycans which inhibited collagen-induced platelet aggregation ex vivo, such as heparin, also enhanced blood loss (Fernandez et al, 1986b). However, glycosaminoglycans which did not inhibit platelet aggregation, such as dermatan sulfate, did not enhance blood loss.
Sulfated glycosaminoglycans, which bind to the vessel wall, may also alter vessel wall permeability, thereby enhancing blood loss. In support of this possibility, Blajchman et al (1989) demonstrated that vessel wall permeability was enhanced when rabbits were treated with heparin. In contrast, vessel wall permeability was unaffected when animals were treated with dermatan sulfate or low molecular weight heparin, both of which were less sulfated than heparin and do not bind to the endothelium.

The above studies focused on the acute bleeding side-effects of dermatan sulfate, however, the bleeding side-effects of a continuous infusion of dermatan sulfate at doses used to prevent thrombus growth are unknown. Similarly the bleeding side-effects of the concurrent administration of either [tPA and dermatan sulfate] or [tPA and heparin] are unknown. This is investigated in this thesis.

VIII. THE TREATMENT OF ARTERIAL THROMBOSIS

1. Clinical use of heparin in the treatment of arterial thrombosis

Heparin has also been used to treat and to prevent arterial thrombotic diseases in the absence of thrombolytic therapy (Chesbro and Fuster, 1986; Serneri et al, 1987; Théroux et al, 1988; Stein et al, 1989; The RISC Group, 1990). To understand the rationale for its use in this situation, it is important to highlight certain fundamental differences
between arterial thrombus formation and venous thrombus formation. Arterial thrombi are formed under conditions of high blood flow and are usually the result of vessel wall injury, such as the rupture of an atherosclerotic plaque or direct endothelial cell damage (Stein et al, 1989). The latter may occur following surgery, such as in patients undergoing coronary artery bypass grafting or angioplasty (Stein et al, 1989; Harker, 1986).

An acutely formed arterial thrombus is comprised mostly of an aggregated platelet mass, interlaced with fibrin (Harker and Slichter, 1972; Harker et al, 1977). Activation and subsequent recruitment of more platelets into this thrombus occurs by several mechanisms. Collagen is exposed following vessel wall injury, and the coagulation cascade may also be activated by the generation of tissue factor, both of which are potent stimuli for further platelet activation (Stein et al, 1989). Platelets which initially adhere to the injured vessel wall may release a number of constituents, some of which promote further platelet aggregation and adhesion, and activate the coagulation cascade (van Aken, 1974). The latter results in subsequent fibrin formation, and fibrin is necessary to consolidate and stabilize the platelet thrombus (van Aken, 1974; Fitzgerald, 1989). Thus, it is conceivable that anticoagulant therapy may inhibit arterial thrombus formation and growth.

In support of this possibility, it has been
demonstrated in a number of studies that heparin treatment alone appears to be beneficial in patients with coronary artery disease compared to untreated patients (Serneri et al, 1987; Théroux et al, 1988; The SCATI Group, 1989). However the benefit of heparin in these patients relative to aspirin treatment remains to be established (Théroux et al, 1988; Hennekens et al, 1989; The RISC Group, 1990). It is possible that antiplatelet drugs provide a better protection than heparin against further vascular sequelae in situations where fibrin plays a relatively minor role. Alternatively, heparin may also be less effective when thrombin is bound to platelets or fibrin deposited at the injured vessel wall site.

2. Experimental use of heparin in the treatment of arterial injury.

Heparin has had varying effects in the prevention of platelet and/or fibrin deposition onto injured vessel walls in experimental models of arterial thrombosis (Harker et al, 1979; Groves et al, 1982; Jang et al, 1990). This variation may be explained, in part, by the different doses of heparin used in these studies. For example, Heras et al (1989) found a dose-dependent inhibition of platelet deposition by heparin after carotid angioplasty in pigs. The dose of heparin required to effectively inhibit platelet deposition generated plasma levels far in excess of those routinely achieved clinically (14 U/ml versus 0.2 to 0.4 U/ml, respectively). It is possible that the effect of heparin at these high doses,
was due to the catalysis of thrombin inhibition by heparin cofactor II, not antithrombin III. Interestingly, hirudin, a specific thrombin inhibitor was more effective than heparin in preventing platelet and fibrin deposition on the injured vessel wall. Thus, these observations indicate that thrombin contributes to the growth of arterial thrombi, and that heparin is a poor inhibitor of thrombus growth, presumably because when thrombin is bound to the injured vessel wall, it is resistant to inhibition by heparin/antithrombin III. The possibility that thrombin is more susceptible to inhibition by dermatan sulfate/heparin cofactor II than to heparin/antithrombin III in the arterial circulation is investigated in this thesis.
AIMS OF PRESENT STUDY

In the preceding pages, a number of issues concerning the use of heparin and dermatan sulfate as antithrombotic agents have been identified. These issues raise a number of questions:

1. Can a better antithrombotic effect be achieved by the catalysis of thrombin inhibition by dermatan sulfate-heparin cofactor II than by heparin-antithrombin III?

2. Can dermatan sulfate more effectively catalyse the inhibition of thrombin bound to a thrombus by heparin cofactor II, than heparin can by catalysing thrombin inhibition by antithrombin III?

3. Does dermatan sulfate or heparin act synergistically with tPA to enhance fibrinolysis? If so, is any beneficial effect with these combinations offset by an enhanced bleeding side-effect?

To address these questions, the following experiments were performed:

1. The pharmacokinetics and pharmacodynamics of dermatan sulfate were determined to allow the appropriate comparisons to be made with heparin.

2. The relative abilities of heparin and dermatan sulfate to inhibit thrombus formation induced by different stimuli were determined.

3. The abilities of dermatan sulfate and heparin to inhibit thrombus growth in the venous circulation were
determined.

4. Any synergism between [heparin and tPA] or [dermatan sulfate and tPA] in effectively reducing thrombus size was determined.

5. Bleeding side-effects between any synergistic effects achieved in (4) with either combination were determined.

6. The abilities of dermatan sulfate and heparin to inhibit thrombus growth in an arterial injury model was determined.
CHAPTER 2: MATERIALS AND METHODS
1. MATERIALS

(a) Glycosaminoglycans and other drugs used in vivo

Unfractionated heparin (Batch 932, derived from porcine mucosa) was obtained from Biosynth B.V., Oss, The Netherlands. It had specific activities of 150 anti-thrombin and anti-factor Xa U/mg.

Dermatan sulfate (derived from porcine mucosa) was obtained from Mediolanum Farmaceutici, Milan, Italy. Two different batches were used, batch 30 (DS30), which had specific activities of 4 anti-thrombin U/mg and <1 anti-factor Xa U/mg; and batch 48 (DS48), which had a specific activity of 2 anti-thrombin U/mg and no detectable anti-factor Xa activity. The specific activity of dermatan sulfate was obtained by reference to the heparin standard (batch 932) using the chromogenic assays described below. The lower specific activities of the two batches of dermatan sulfate implies that for equivalent anti-thrombin units, the gravimetric amount of dermatan sulfate will be greater than heparin.

tPA (batch 103796-1) obtained from Smith, Kline and French, Swedeland, PA, had a specific activity of 575,000 IU/mg.
(b) Other anticoagulants

Other agents used as anticoagulants were: 4% acid citrate (0.1 M citric acid and 0.1 M trisodium citrate), 1 part acid citrate : 9 parts whole blood; 3.2% sodium citrate (0.1 M trisodium citrate), 1 part sodium citrate : 9 parts whole blood; and acid citrate dextrose (0.08 M trisodium citrate, 0.07 M citric acid, 0.11 M dextrose), 1 part acid citrate dextrose : 6 parts whole blood. All chemicals used to prepare these solutions were obtained from Sigma Chemical Co., St. Louis, MO.

(c) Anesthetics

Ketamine hydrochloride (100 mg/ml) was obtained from Rogaar/STB Inc., London, ON. Acepromazine maleate (Atravet, 25 mg/ml) was obtained from Ayerst Laboratories, Montreal, PQ. Sodium pentobarbital (Somnotol, 65 mg/ml) was obtained from MTC Pharmaceuticals, Hamilton, ON.

(d) Anticoagulant assay materials

The chromogenic substrates, S-2222 (CBz-Ile-Glu)γOR-(Gly-Arg-pNA-HCl), S-2238 (H-D-Phe-Pip-Arg-pNA), S-2251 (H-D-Val-Leu-Lys-pNA) and plasmin (25 casein U/vial) were obtained from Helena Laboratories, Beaumont, TX. Human thrombin (100 NIH U/vial) and bovine factor Xa (100 μg/vial) were obtained from Interhaematol, Hamilton, ON. Microtitre plates were obtained from Flow Laboratories, Inc., McLean, VA.
(e) **Prothrombotic stimuli**

Human brain tissue thromboplastin was obtained from the National Reference Laboratory for Anticoagulant Control, Worthington Hospital, Manchester, UK. Bovine thrombin was obtained from Parke-Davis, Mississauga, ON. Purified human factor Xa was prepared by and obtained from Dr. F.A. Ofosu (1981). Purified human prothrombin (1.1 mg/ml) was obtained from Enzyme Research Laboratories, Inc., South Bend, IN.

(f) **Rabbits**

All studies were performed using New Zealand White rabbits of either sex, with an average weight of 2.75 kg. The rabbits were anesthetized by a standard procedure. Each rabbit was injected intramuscularly with 2 ml of ketamine, in conjunction with 1 ml of AtravetR. Anesthesia was maintained as needed by administration of bolus intravenous injections of sodium pentobarbital (diluted 1/2 with saline).

All experiments were performed in a blinded fashion, with every treatment assigned randomly. This was achieved by one person preparing the different treatments and a second person injecting the treatments and performing the experiments.

2. **METHODS**

(a) **Processing of blood samples**

All blood samples were collected via a carotid artery cannula (Intramedic tubing, PE-190) in all of the animal
experiments, unless otherwise stated. Blood samples for anticoagulant assays were collected into the appropriate anticoagulant and immediately centrifuged at 1800 g for 10 minutes to prepare platelet poor plasma. The plasma sample was then transferred to a 1.5 ml Eppendorf tube, and stored on ice until each experiment was completed. Then all of the plasma samples were frozen and stored at -40°C until they were assayed.

All blood samples for radioactivity measurements were collected in 1 ml tuberculin syringes containing no anticoagulant, and transferred to 12 x 75 mm plastic counting tubes. The radioactivity of each sample was then determined using a Beckman 4000 Gamma Counter.

(b) Anticoagulant parameters

(i) Measurement of anticoagulant levels ex vivo

The anticoagulant levels achieved ex vivo after the administration of both dermatan sulfate and heparin were measured using chromogenic assays. It was difficult to accurately compare the anticoagulant effects of heparin and dermatan sulfate to each other in ex vivo plasma, since heparin and dermatan sulfate exert their anticoagulant effects via different inhibitor pathways. Thus assays which are sensitive measures of either heparin or dermatan sulfate levels need to optimize either the antithrombin III- or heparin cofactor II-mediated pathway, but cannot optimize both
(Dupouy et al, 1988; Teien et al, 1976; Yin et al, 1973). However, the chromogenic assays were chosen for the measurement of anticoagulant activity *ex vivo* for several reasons. First, these chromogenic assays are used clinically to monitor heparin levels, thus this allowed a comparison between the anticoagulant levels achieved during the experiments in this thesis to those achieved clinically. Second, since the doses of dermatan sulfate and heparin were administered in anti-thrombin units in the experiments in this thesis, these assays were used to determine if the doses which were administered to rabbits achieved the expected circulating anticoagulant levels.

The major limitations with chromogenic assays include, i) their lack of sensitivity, since anticoagulant levels below 0.05 anti-thrombin or anti-factor Xa U/ml are not detectable; and ii) the necessity to dilute the test plasma in order to perform the assay. This dilution of *ex vivo* plasma is required since the heparin or dermatan sulfate catalysed inhibition of factor Xa and/or thrombin proceeds too quickly to be quantified in undiluted plasma. However, dilution alters the concentration of endogenous inhibitors (antithrombin III and heparin cofactor II) present in plasma which are required for the heparin- and dermatan sulfate-induced catalysis of either factor Xa and/or thrombin inhibition (Fernandez et al, 1987). These dilutions will
favour antithrombin III-catalysed reactions since endogenous antithrombin III concentrations are higher than heparin cofactor II concentrations. Therefore, when comparing the anti-thrombin activity of dermatan sulfate and heparin based on these chromogenic assays, the activity of dermatan sulfate will probably be somewhat underestimated.

(ii) **Anti-factor Xa assay chromogenic assay**

The anti-factor Xa levels achieved by heparin and dermatan sulfate *ex vivo* were measured chromogenically using a modified method of Teien et al (1976). *Ex vivo* samples were diluted with pooled rabbit plasma to achieve an expected concentration of heparin or dermatan sulfate between 0.1 - 0.5 U/ml of plasma. The chromogenic assay was performed in 96-well microtitre plates at 37°C. To prepare a standard curve, known amounts of heparin or dermatan sulfate were assayed on each microtitre plate simultaneously with the *ex vivo* samples. Factor Xa was diluted (usually between 1/50 - 1/60) in Michaelis buffer (0.035 M sodium acetate, 0.036 M sodium barbital, 0.14 M NaCl) containing 1% BSA, so that a non-heparinized plasma sample had an optical density of 0.6 - 0.7 (405 nm, visible light, blue filter).

Each plasma sample (standard curve or test sample) was diluted 1/9 with chromogenic buffer (0.2 M Tris, 1.0 M HCl, 0.25 M K₂EDTA, 0.24 M NaCl). Duplicate 75 μl aliquots of this mixture were transferred to the wells in the microtitre
plate, which had been prewarmed to 37°C. The diluted plasmas were warmed for 2 minutes, and then 40 μl of factor Xa was added to each well. After a 90 second incubation, 75 μl of S-2222 (1 mM) was added and the mixture was incubated for another 5 minutes. The reaction was then terminated by adding 125 μl of 50% acetic acid. The optical density of the samples were determined using a Titertek Multiskan spectrophotometer at 405 nm. A standard curve was derived for each plate, using a linear regression analysis. The amount of anti-factor Xa activity generated ex vivo by heparin or dermatan sulfate in each sample was determined by extrapolation from the standard curve.

(iii) Anti-thrombin assay chromogenic assay

The anti-thrombin levels achieved by heparin and dermatan sulfate ex vivo were also measured chromogenically (Yin et al, 1973). Thrombin was diluted (usually between 1/10 - 1/20) so that a non-heparinized plasma sample has an optical density of 0.6 - 0.7 at 405 nm. Each plasma sample (standard curve or test sample) was diluted 1/5 with chromogenic buffer. Duplicate 75 μl aliquots of this mixture were transferred to the wells in the microtitre plate, which had been prewarmed to 37°C. The diluted plasmas were warmed for 2 minutes at 37°C, then 25 μl thrombin was added to each well. After a 60 second incubation, 75 μl S-2238 (0.75 mM) was added and 2 minutes later, the reaction was terminated by adding 125 μl
50% acetic acid. The ex vivo test samples were quantitated as described for the anti-factor Xa assay. Examples of typical standard curves for both assays are shown in Figure 6.

(iv) **α2-Antiplasmin assay**

The effects of tPA on systemic fibrinolysis was measured chromogenically by the method of Teger-Nielsson et al (1977). The assay was performed at 37°C in 96-well microtitre plates. A standard curve was prepared by diluting pooled rabbit plasma into Tris-HCl buffer (0.04 M Tris-HCl, 0.05 M Tris, 0.1 M NaCl, pH 7.4). Each ex vivo test sample was diluted 1/30 in buffer, and 150 μl of the standard curve and test samples were added to the microtitre plate (in duplicate). The diluted plasma was incubated for 2 minutes at 37°C. Then 60 μl plasmin (0.25 CU/ml) was added. One minute later, 50 μl S-2251 (3 mM) was added, and 3 minutes later the reaction was terminated by adding 50 μl of 50% acetic acid. The optical density of the samples were measured at 405 nm and ex vivo test samples were quantitated against the standard curve.

(v) **Plasma fibrinogen levels**

The circulating plasma fibrinogen levels were determined using the clotting assay described by van Clauss (1957). The amount of fibrinogen present in ex vivo plasma samples was determined by comparing them to pooled rabbit plasma that had previously been assayed using the clottable
Figure 6: Typical in vitro standard curves obtained for heparin (A), DS48 (B) and DS30 (C) using the chromogenic assays for anti-thrombin and anti-factor Xa activity. $r$ represents the correlation coefficient and $m$ represents the slope of the line.
fibrinogen assay. Plasma containing known amounts of fibrinogen was diluted (1/5 - 1/40) in Veronal buffer (0.01 M trisodium acetate, 0.01 M sodium diethyl barbituate, 0.15 M NaCl, 0.005 M HCl, pH 7.4) to obtain a standard curve. Duplicate test samples were diluted 1/10 and 1/20 in Veronal buffer. The assay was performed on a Becton-Dickinson Fibrometer (FibroSystem\textsuperscript{a}). 200 µl of test sample or standard curve sample was prewarmed for 2 minutes to 37°C, then 100 µl of thrombin (Parke-Davis, 2 U) was added to the plasma in a Fibrometer cup and the timer was started. The clotting time was recorded and compared to the standard curve to determine the mg% of fibrinogen in the plasma. Normal fibrinogen levels in rabbits range from 200-400 mg%.

(c) Preparation of radiolabelled dermatan sulfate

(i) Coupling dermatan sulfate to SHPP

Dermatan sulfate was coupled to 3-(4-hydroxyphenyl)propionic acid N-hydroxysuccinimide ester (SHPP) using the method of Dawes and Pepper (1979). First, 50 mg of dermatan sulfate (batch 30) was mixed with 5 mg SHPP and incubated for 20 hours at 4°C. This mixture was then passed over a 30 x 1 cm G-25 Sephadex column eluted with 0.15 M NaCl, 0.05 M phosphate buffer (PB), to separate SHPP bound to dermatan sulfate from unbound SHPP. The fractions containing the SHPP-dermatan sulfate were then added to a CNBr-activated Sepharose 4B column coupled with protamine sulfate. SHPP-dermatan
sulfate was recovered after elution with 1 M NaCl, 0.05 M PB, and the dermatan sulfate content of these fractions was measured using a protamine sulfate assay (described below), and for SHPP levels by UV spectrometry. The fractions containing SHPP and dermatan sulfate were then pooled. The final SHPP-dermatan sulfate concentration was 10 mg, which was diluted to 1 mg/ml final concentration. SHPP incorporation was determined by UV spectrometry, with a final recovery of -1 mg SHPP (extinction coefficient for SHPP, $\epsilon_{280} = 4.55 \times 10^3$). SHPP-dermatan sulfate was biologically active as determined by the anti-thrombin chromogenic assay.

(ii) Radiolabelling SHPP-dermatan sulfate

SHPP-dermatan sulfate was radiolabelled with $^{125}$I, using the chloramine T method (Greenwood et al, 1963). First, SHPP-dermatan sulfate (100 µg) was mixed with 0.4 M phosphate buffer, and then mixed for 1 minute with 20 µl of $^{125}$I (3 mCi) and 20 µl chloramine T (5 mg/ml). The reaction was then stopped by adding Na$_2$S$_2$O$_5$ (7 mg/ml) and the carrier KI (2 mg/ml) was then added. The mixture was then passed over a 30 x 1 cm G-25 Sephadex column to separate the $^{125}$I-SHPP-dermatan sulfate from unbound $^{125}$I. The final specific activity was routinely -10 µCi/µg SHPP-dermatan sulfate. The molecular weight profile of $^{125}$I-SHPP-dermatan sulfate was compared to the starting material using gel filtration (50 x 1 cm Sephadryl S-200 column).
(iii) Protamine sulfate assay

To determine the concentration of dermatan sulfate present in each fraction eluted from the different columns, a protamine sulfate assay was used as described by Hatton et al (1978). A standard curve was prepared by diluting a DS30 solution to between 0 - 250 μg/ml in a Tris-saline buffer (0.02 M Tris, 0.15 M NaCl, pH 7.4). Each test sample (125 μl from each fraction) or standard curve sample was incubated with 50 μl of protamine sulfate (1 mg/ml) for 10 minutes, resulting in DS30-protamine complex formation. After 10 minutes, 250 μl of 0.1 M L-arginine hydrochloride and 575 μl of 0.02 M Tris (pH 7.8) were added to each sample to prevent precipitation of the complexes. The optical density of each sample was then measured at 470 nm (visible light). The amount of DS30 in each fraction was calculated from the standard curve.

(d) Preparation of radiolabelled fibrinogen

(i) Fibrinogen purification

Rabbit fibrinogen was prepared according to the method described by Regoezzi (1970). Eight rabbits were exsanguinated and the blood was collected into acid citrate dextrose. The blood was centrifuged at 1800 g for 15 minutes, to prepare platelet poor plasma. The platelet poor plasma from all the rabbits was then pooled. All successive centrifugations were at 1800 g for 10 minutes unless otherwise stated. One gram
of BaSO₄ for every 10 ml of plasma was slowly added. This mixture was then centrifuged for 30 minutes at 1800 g. The supernatant was decanted to a second tube and an equal volume of 0.15 M NaCl was added. Saturated ammonium sulfate (NH₄)₂SO₄ was then added dropwise (using a peristaltic pump) to 17% saturation in one hour. The solution was centrifuged and the low solubility fibrinogen precipitate discarded. More ammonium sulfate was added, again dropwise for another hour to achieve 24% saturation. The solution was centrifuged and the supernatant discarded. The precipitated fibrinogen was pooled and washed several times with ammonium sulfate saturated to 24%. The fibrinogen was then dissolved in 0.005 M citrate saline and dialysed until free of ammonium sulfate. After dialysis, the purified fibrinogen was stored at 4°C until it was assayed for purity and clottability.

(ii) Clottable fibrinogen assay

The above fibrinogen solution was assayed using a clottable protein assay (Jakobsen and Kierulf, 1973). The purified fibrinogen solution was diluted with an equal volume of distilled water (in triplicate). This diluted fibrinogen was then clotted by the addition of 10 µl 0.02 M CaCl₂ and 10 µl 1000 U/ml Parke-Davis thrombin. After incubation for 60 minutes at 22°C, the thrombus was removed from the solution with a wooden applicator stick. The optical density of the remaining supernatant was determined at 280 nm, and compared
to the optical density of a sample of purified fibrinogen. The percent clottable fibrinogen was calculated by determining the ratio of the optical density of the supernatant to the optical density of the purified fibrinogen. The concentration of the purified fibrinogen was determined from the optical density at 280 nm, using the extinction coefficient $E^{11}_{1}=16$. Clottability ranged from 80-90%, and the final concentration of fibrinogen was 10.3 mg/ml, which was divided into 1 ml aliquots and stored at -40°C.

(iii) Labelling purified fibrinogen

A modified method of McFarlane (1958) was used to label the purified fibrinogen. Four 1 ml aliquots of purified fibrinogen were used (10 mg/ml). To each aliquot was added 0.7 ml alkaline glycine buffer, 1.2 μCi Na$^{125}$I and 10 μl of iodine monochloride. Each aliquot was mixed well, then passed over an anion exchange column to remove the free $^{125}$I. The column was previously equilibrated with phosphate buffer (pH 7.3). The pooled aliquots were collected in one fraction, and then diluted in phosphate buffer until the concentration was 100 μCi/ml. Purified fibrinogen was bound to 98% of the $^{125}$I as determined by trichloroacetic acid (TCA) precipitation. The $^{125}$I-fibrinogen was stored frozen at -40°C until needed.

(e) Radiolabelling Prothrombin

Purified human prothrombin (1.1 mg/ml) was radiolabelled with Iodogen® (Pierce Chemical Co., Rodeford,
A glass vial was coated with Iodogen® by dissolving 5 μg of Iodogen® in 100 μl of chloroform, which was the added to the borosilicate glass vial. The chloroform was then evaporated to dryness. Human prothrombin (100 μg) was mixed with 5 μl NaI (1 μg/ml), 50 μl sodium borate buffer (0.2 M sodium borate, 0.15 M NaCl, pH 8.0) and 1 mCi of Na¹²⁵I. This mixture was then added to the Iodogen®-coated vial and allowed to react for 1 minute at 25°C with continual stirring. The reaction was stopped by removing the mixture from the Iodogen®-coated vial, and then placed on a 15 x 1 cm G-25 Sephadex column to separate radiolabelled prothrombin from unbound ¹²⁵I. The G-25 Sephadex column was pre-loaded with 0.01 M PB containing 0.1% BSA to prevent non-specific prothrombin binding and then eluted with 0.01 M PB. The specific activity was 176 μCi/100 μg prothrombin, with 97% of the radioactivity incorporated into the protein (by TCA precipitation). The biological activity of prothrombin was unchanged compared to the starting material as determined by activation of radiolabelled prothrombin by Taipan snake venom.

(f) Preparation of ⁵¹Cr-labelled platelet suspension

Four donor rabbits for every 6 recipient rabbits were anesthetised with sodium pentobarbital (30 mg/kg) via the marginal ear vein. The left carotid artery was cannulated and each donor rabbit was exsanguinated. The blood was collected into acid citrate dextrose. The blood was then centrifuged
at 1000 rpm (200 g) for 15 minutes at 25°C to obtain platelet rich plasma (PRP). Blood from one rabbit was collected into sodium citrate. This blood was centrifuged at 2800 rpm (1800 g) for 30 minutes at 25°C to obtain platelet poor plasma (PPP). The PPP was then transferred to a new tube and stored at 4°C until needed.

The PRP was transferred into new tubes and centrifuged at 2800 rpm (1800 g) to obtain a platelet pellet. The PPP was aspirated, and the platelet pellets were resuspended in calcium-free Tyrodes albumin (0.35%), pH 6.25, containing 100 μl of 2% EGTA. The platelet suspension was then incubated with 150 μCi of Na\textsuperscript{51}CrO₄ per recipient rabbit for 40 minutes. The platelet suspension was centrifuged (1800 g for 10 min) and resuspended in the calcium-free Tyrodes to wash the platelets. The platelets were centrifuged again (1800 g for 10 min) and the final platelet pellet was resuspended in the sodium citrated PPP, which had been prewarmed to 37°C. A final platelet count and radioactivity determination was performed to determine the specific activity (typical recovery: 1500 plt/CPM, 3 x 10⁹ plts/3 ml, < 2% red cell contamination). The final suspension was drawn into 3 ml syringes and injected into each recipient rabbit.

(g) **Statistical Analysis**

Data were analyzed using a one-way analysis of variance and the appropriate multiple group comparison test
(Bruning and Kintz, 1977; Rosner, 1982).

3. **EXPERIMENTAL DESIGNS**

   (a) **Determination of the distribution and clearance half-lives of dermatan sulfate**

   The distribution (α) and clearance (β) half-lives of $^{125}$I-dermatan sulfate (DS30) were measured in rabbits injected with increasing doses of $^{125}$I-SHPP-DS30, ranging from 0.01 to 40 U/kg. First, rabbits were anesthetized and then the left carotid artery of each rabbit was isolated and cannulated. Each rabbit was injected with sodium iodide (4 mg/kg) to prevent any uptake of $^{125}$I by the thyroid. Five minutes later, the rabbits were injected with the radiolabelled DS30 via the marginal ear vein. Serial blood samples (3 ml) were collected from the carotid artery over 4 hours. A 500 μl subsample was transferred to a counting tube to measure the radioactivity. The remaining blood was centrifuged (1700 g for 15 min) to prepare platelet poor plasma, which was then assayed for its anti-thrombin activity. At the end of the experiment all the animals were euthanized with an overdose of pentobarbital.

   The radioactivity (CPM) determined at 1 minute was arbitrarily expressed as 100%. The radioactivity determined at all subsequent time points was expressed as a percent of the 1 minute sample. Similarly the anti-thrombin activity obtained at 1 minute was arbitrarily expressed as 100%. This allowed a direct comparison between the clearance of the
radiolabelled DS30 molecules and the distribution and clearance of its anti-thrombin activity.

(i) Calculation of the $\alpha$, $\beta$ and $\gamma$ half-lives of DS30

The curve was divided into three exponentials: an $\alpha$-phase or distribution phase (which was measured between 1 and 10 minutes), a $\beta$-phase or clearance phase (which was measured between 10 and 45 minutes) and a $\gamma$-phase or residual phase, (which was measured between 45 minutes and 4 hours). The residual background or $\gamma$-phase of dermatan sulfate was calculated first, by establishing a linear regression line of the data points between 45 minutes and 4 hours. The line of best fit was extended back to the y-axis, and this line was subtracted from the values obtained between 10 and 45 minutes. These differences were then used to calculate a second linear regression line, which was again extended back to the y-axis, to calculate the clearance half-life ($t_{1/2} \beta$). The differences between the $t_{1/2} \beta$ regression line and the data points from 1 minute to 10 minutes were then used to calculate the distribution half-life ($t_{1/2} \alpha$). This is illustrated diagrammatically in Figure 7.

(b) Prevention of venous thrombosis

Rabbits were anesthetized, and a 1.5 cm length of each jugular vein was isolated between two loose sutures. The left carotid artery was cannulated. Each rabbit was injected with 100 $\mu$l of $^{125}$I-fibrinogen (containing 1 mg and 10 $\mu$Ci),
Figure 7: The clearance curve of radioactivity after a bolus injection of 10,000 μg/kg. The curve is divided into three exponentials, (r > 0.96 for each curve) from which the α, β and γ half-life was calculated.
followed 10 minutes later by the glycosaminoglycan treatment. Three minutes later, a blood sample was collected, and the rabbits were injected immediately with the thrombotic stimulus, i.e. thrombin (12.5 U/kg), factor Xa (20 μg/kg) or tissue thromboplastin (1 mg/kg), given over 30 seconds via the carotid artery cannula. Thirty seconds after this, both jugular vein sutures proximal to the heart were tightened, allowing each vein segment to fill with blood. The distal sutures were then tightened. Stasis was maintained for 15 minutes. The isolated jugular vessels were then slit open longitudinally and any formed thrombus was removed. The radioactivity of each thrombus was measured and its size expressed in μls of blood, based upon the circulating whole blood radioactivity (Carter et al, 1982).

(c) **Treatment of venous thrombosis**

(i) **Prevention of $^{125}$I-fibrin accretion**

Rabbits were anesthetized and the left carotid artery was cannulated. Both jugular veins were exposed and a 2 cm segment was isolated on either side. Each jugular vein segment was emptied of blood, and blood flow was temporarily occluded by proximal and distal clamps. One half ml of whole blood was collected from the carotid artery cannula into a tuberculin syringe containing 1 U (50 μl) of thrombin (Parke-Davis bovine thrombin). One hundred and fifty μl of the clotting blood was immediately injected back into each
isolated venous segment via a 25 gauge needle. Fifteen minutes later, a 10 cm length of 3-0 suture was passed longitudinally through the vessel wall and the forming thrombus to prevent the thrombus from embolizing once blood flow was restored 15 minutes later. Each animal was then injected with 100 μl $^{125}$I-fibrinogen via the carotid artery cannula. Rabbits were then injected with increasing doses of either heparin, dermatan sulfate or saline, given either as an infusion or as a bolus injection, via the marginal ear vein (see Figure 8 for a schematic illustration of the experimental design). Four hours later, the venous segments containing the thrombi were tied off, slit open longitudinally, and the remaining thrombi removed. The specific activity of fibrinogen in circulating whole blood was estimated from the mean radioactivity of blood samples collected at hourly intervals over the four hour period. The ratio of the radioactivity of the thrombus to the circulating fibrinogen radioactivity was used to estimate fibrin accretion, which was expressed as μg of $^{125}$I-fibrin accreted onto the thrombus (Chiu et al, 1977).

(ii) Prothrombin clearance and accretion

Prothrombin clearance was measured over 30 minutes by injecting radiolabelled prothrombin into rabbits and measuring the disappearance of $^{125}$I from the circulation in the presence and absence of existing thrombi. Thrombi were made in the
A. INFUSION TREATMENT

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30'  1  2  3  4  hours

FORM THROMBI
- RE-ESTABLISH BLOOD FLOW
- INJECT 125I-FIBRINOGEN
- COMMENCE INFUSION

B. BOLUS TREATMENT

| BOLUS |

| FORM THROMBI |
| RE-ESTABLISH BLOOD FLOW |
| INJECT 125I-FIBRINOGEN |
| COMMENCE INFUSION |

Figure 8: A schematic representation of the experimental design used for the treatment of venous thrombosis, highlighting the differences between infusion and bolus treatment.
jugular veins of rabbits using the same method described for the treatment of venous thrombosis. Sham-operated animals underwent the same surgical procedures except no thrombi were formed in the jugular veins. Rabbits were injected with increasing doses of either DS48, heparin or saline. Five minutes later, the animals were injected with 50 μl of $^{125}$I-prothrombin (80 μg, 6 x $10^7$ CPM) and serial blood samples (1 ml, no anticoagulant) were collected before and 1, 3, 5, 7, 10, 15 and 30 minutes after injection of the prothrombin. At 30 minutes the thrombi were removed from the isolated jugular vein segments, and the amount of $^{125}$I-(pro)thrombin accreted was determined, based upon the circulating whole blood $^{125}$I-prothrombin specific activity and thrombus radioactivity. $^{125}$I-prothrombin clearance was expressed as a percentage of the 1 minute counts, which were arbitrarily expressed as 100%.

Similar experiments were performed with $^{125}$I-albumin (50 μl, 3 μCi/80 μg) to determine non-specific protein accretion onto the thrombus.

(d) Venous fibrinolysis model

Fibrinolysis by tPA, in the presence or absence of dermatan sulfate or heparin, was measured as the disappearance of $^{125}$I-fibrin from existing thrombi over a 4 hour period. Rabbits were anesthetized as described previously, a 2 cm segment of both jugular veins was exposed and the left carotid artery was cannulated. Blood flow in the jugular vein was
temporarily occluded, by clamping the segment distally to the heart, emptying it of blood and then clamping the segment proximally. Ten ml of blood (drawn into sodium citrate) had previously been obtained from one of the rabbits and to this was added 200 μl (20 μCi) of 125I-fibrinogen stock solution. The specific activity of the radiolabelled blood was then determined by removing an aliquot and counting the radioactivity. One half ml of this radiolabelled anticoagulated blood was drawn into a tuberculin syringe containing 1 U (50 μl) of thrombin (Parke-Davis bovine thrombin) and 20 μl of 0.25 M CaCl₂. One hundred and fifty μl of clotting blood was then injected into each isolated venous segment via a 25 gauge needle. Fifteen minutes later, a 10 cm length of 3-0 suture was passed longitudinally through the forming thrombus and the vessel wall to prevent the thrombus from embolizing once blood flow was restored 15 minutes later. The test compounds were then administered via the marginal ear vein, with 10% of the total dose given as a bolus, and the subsequent 90% of the dose given as a continuous 4 hour infusion. Four hours later, the venous segments containing the thrombi were tied off, slit open longitudinally, and any remaining thrombi were removed. The thrombi were then counted on a gamma counter. The ratio of the radioactivity of the thrombus (CPM) to the specific activity of the initially injected blood (CPM/μl) in each
jugular segment, was used to quantitate the amount of lysis that occurred over 4 hours. Blood samples were collected at hourly intervals to determine anticoagulant and ατ-antiplasmin levels.

(e) Hemorrhagic side-effects

Blood loss was measured using a modification of the method by Carter et al (1982). The rabbits were anesthetized as previously described and the left carotid artery was cannulated. The loading dose of either DS48, DS30, heparin or saline was then injected via the marginal ear vein. Five standard cuts were made in one of the rabbit ears by puncturing the microvascular area with a Bard-Parker #11 scalpel blade. The ear was then immersed into a 1 litre water bath, which had been prewarmed to 37°C. The four hour infusion of the remaining 90% of the dose was then started. The amount of blood lost from these 5 cuts into the water bath was determined at 30 minute intervals by removing a 1 ml aliquot and measuring the amount of colour generated from the lysed red cells in the hypotonic solution. Serial blood samples for anticoagulant levels were collected prior to treatment, and at hourly intervals after treatment.

To determine the amount of blood lost into the water bath, the optical density (405 and 492 nm) of 1 ml aliquots of the water bath were measured, and compared to the optical density of a standard curve, derived by diluting known amounts
of whole blood in distilled water.

(f) **Continuous arterial injury**

Rabbits were anesthetized and the right carotid artery was isolated under aseptic conditions. The initial bolus loading dose of each treatment was then injected via the marginal ear vein. A 9 cm sterile cannula was then inserted into the isolated carotid artery, so that the tip of the cannula was situated between the aortic valves and the carotid orifice. The cannula was then tied in place with a sterile 3-0 silk suture. In sham-operated animals (i.e. with no cannula), the right carotid artery was isolated and tied off. The incisions were then sutured closed. Fifteen minutes after cannula placement or tying off the carotid artery, the rabbits were injected with 3 ml of $^{51}$Cr-labelled platelets ($2.5 \times 10^9$ plt/ml) and 100 μl of $^{125}$I-fibrinogen. Blood samples (3 ml) were collected from each rabbit via the marginal ear vein at 4, 20, 28 and 44 hours. One half ml of this blood sample was used to quantify circulating radioactivity levels and the remaining 2.5 ml was centrifuged to obtain platelet poor plasma, which was assayed later to determine anticoagulant levels. Dermatan sulfate, heparin and saline were injected every 8 hours and aspirin was injected daily via the marginal ear vein. After 48 hours, the rabbits were again anesthetized as previously described and a cannula was inserted in the right jugular vein. The final 48 hour blood sample was taken
via the jugular vein cannula, and an additional 3 ml blood sample was taken to determine the hematocrit and then centrifuged for platelet rich plasma.

The rabbits were then heparinized (200 U/kg) and 2 minutes later were euthanized. A longitudinal cut was made in the right carotid artery and arch of the aorta, as far as the left ventricle, and the position of the indwelling cannula was recorded. The artery segment surrounding the cannula tip was then removed and washed with plain tyrodes to remove any excess blood. The artery segment (lumen side up) was placed on a rubber pad and the thrombus and artery wall were excised using a #6 cork-hole borer. The excised segments were washed in plain Tyrodes and placed in a vial for gamma counting.

Platelet and fibrin deposition were calculated using the ratio of $^{51}$Cr and $^{125}$I counts on the artery segment as compared to the mean whole blood radioactivity over 48 hours. Platelet half-life was calculated using the circulating $^{51}$Cr count over the 48 hour period, with 100% recovery at 4 hours (the time needed for equilibration of the injected platelets).
CHAPTER 3: RESULTS
I. PHARMACOKINETICS AND PHARMACODYNAMICS OF DERMATAN SULFATE

1. Pharmacokinetics

The molecular weight of DS30 was unchanged when it was coupled with SHPP and then labelled with $^{125}$I (Figure 9). The anti-thrombin activity of the $^{125}$I-SHPP-DS30 was also similar to the starting material, as measured using a chromogenic assay.

Clearance of $^{125}$I-SHPP-DS30 followed a curvilinear pattern, consistent with a two-compartment model (Figure 10). The distribution half-life ($\alpha$-phase) ranged between 6.8 and 9.0 minutes; the clearance half-life ($\beta$-phase) ranged between 23 and 27 minutes; and the residual half-life ($\gamma$-phase) or clearance of the remaining 5% of the dose, ranged between 147 and 266 minutes (Table 1). The half-life of each phase was independent of the dose administered.

2. Pharmacodynamics

The ex vivo anti-thrombin levels generated after administration of $^{125}$I-SHPP-DS30 are illustrated in Figure 11. There was no detectable anti-thrombin activity ex vivo as measured chromogenically following the administration of doses <4 U/kg. There was a dose-related increase in anti-thrombin activity when $^{125}$I-SHPP-DS30 was injected in doses of 4 to 40
Figure 9: Elution of $^{125}$I-SHPP-DS30 and native DS30 from a Sephacryl S-200 column.
Figure 10: The clearance curve of radioactivity after a bolus injection of 40 U/kg of $^{125}$I-SHPP-DS30, plotted semilogarithmically.
Table 1: The α-, β- and γ-half-lives of increasing doses of $^{125}$I-SHPP-DS30 after an intravenous bolus injection.

<table>
<thead>
<tr>
<th>DOSE (µg/kg)</th>
<th>Anti-thrombin U/kg</th>
<th>HALF-LIFE α (minutes)</th>
<th>β (minutes)</th>
<th>γ</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.01</td>
<td>7.0</td>
<td>23.9</td>
<td>208.3</td>
</tr>
<tr>
<td>5</td>
<td>0.02</td>
<td>6.8</td>
<td>24.0</td>
<td>196.7</td>
</tr>
<tr>
<td>10</td>
<td>0.04</td>
<td>6.8</td>
<td>25.0</td>
<td>179.8</td>
</tr>
<tr>
<td>50</td>
<td>0.20</td>
<td>8.0</td>
<td>23.2</td>
<td>146.5</td>
</tr>
<tr>
<td>100</td>
<td>0.40</td>
<td>7.5</td>
<td>25.6</td>
<td>154.2</td>
</tr>
<tr>
<td>500</td>
<td>2.00</td>
<td>8.8</td>
<td>24.7</td>
<td>256.7</td>
</tr>
<tr>
<td>750</td>
<td>3.00</td>
<td>8.1</td>
<td>27.0</td>
<td>266.8</td>
</tr>
<tr>
<td>1000</td>
<td>4.00</td>
<td>8.2</td>
<td>26.9</td>
<td>264.8</td>
</tr>
<tr>
<td>2500</td>
<td>10.00</td>
<td>8.4</td>
<td>26.2</td>
<td>217.4</td>
</tr>
<tr>
<td>5000</td>
<td>20.00</td>
<td>9.0</td>
<td>26.2</td>
<td>203.3</td>
</tr>
<tr>
<td>10000</td>
<td>40.00</td>
<td>8.4</td>
<td>24.6</td>
<td>188.5</td>
</tr>
</tbody>
</table>

Data are expressed as the mean, SD < 5%, n = 6.
Figure 11:  The anti-thrombin activity measured *ex vivo* after a single intravenous injection of doses of DS30 ≥ 4 U/kg. The data are expressed as the mean. SEM ≤ 0.03 U/ml, n = 5.
U/kg. The clearance half-lives of the 20 and 40 U/kg doses were 23.2 and 24.8 minutes respectively. These values were similar to the clearance half-lives obtained with the radiolabelled compound.

These data indicate that clearance of the anti-thrombin activity of DS30 parallels the clearance of the radiolabelled compound.

II. EFFECTS OF HEPARIN AND DERMATAN SULFATE IN THE PREVENTION OF VENOUS THROMBOSIS

To compare the abilities of dermatan sulfate and heparin to prevent thrombus formation induced by different stimuli, the following experiments were performed. Increasing doses of dermatan sulfate (batches DS30 and DS48) and heparin were injected into rabbits. This was followed by the thrombotic stimulus, and thrombus formation was induced using the venous stasis/hypercoagulation technique as previously described. Both dermatan sulfate and heparin were administered as anti-thrombin U/kg in the experiments discussed below. The reader is reminded that i) dermatan sulfate has no anti-factor Xa activity, and ii) the anti-factor Xa activity of heparin is the same as the anti-thrombin activity.

1. **Tissue thromboplastin-induced thrombus formation**

The mean thrombus size in saline-treated animals was
88.5 ± 9.6 μl when tissue thromboplastin (1 mg/kg) was used as the thrombotic stimulus (Table 2). This value is expressed as 100.0 ± 10.8% (mean ± SEM) in Figure 12 (saline control, hatched area). When animals were treated with increasing doses of heparin (2.5 - 10.0 U/kg), there was a dose-related inhibition of thrombus formation. Ten U/kg of heparin inhibited thrombus formation by -75% (p < 0.01). When the animals were treated with increasing doses of dermatan sulfate (either batch 30 or 48), there was also a dose-related inhibition of thrombus formation. The doses of DS30 and DS48 required to achieve the same inhibitory effect as heparin were 3.0 and 1.5 U/kg respectively. Similar observations were obtained when thrombin was used as the initiating stimulus.

2. Thrombin-induced thrombus formation

The mean thrombus size in saline-treated animals was 95.0 ± 12.1 μl when thrombin (12.5 U/kg) was used as the thrombotic stimulus (Table 2). Thus, the size of the thrombus formed in the animals injected with thrombin was similar to the thrombus size formed in animals when tissue thromboplastin was used as the thrombotic stimulus. This value is also expressed as 100.0 ± 12.7% in Figure 13 (saline control, hatched area). When animals were treated with increasing doses of heparin (2.5 - 10 U/kg), thrombin-induced thrombus formation was not inhibited to the same extent as when tissue thromboplastin was the thrombotic stimulus. Ten U/kg of
Table 2: Thrombus size in untreated animals injected with tissue thromboplastin, thrombin or factor Xa. Data are expressed as the mean ± SEM, n=12.

<table>
<thead>
<tr>
<th>Initiating Stimulus</th>
<th>Thrombus Size (μl clot equivalent)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tissue thromboplastin (1 mg/kg)</td>
<td>88.5 ± 9.6</td>
</tr>
<tr>
<td>Thrombin (12.5 U/kg)</td>
<td>95.0 ± 12.1</td>
</tr>
<tr>
<td>Factor Xa (20 μg/kg)</td>
<td>96.9 ± 11.2</td>
</tr>
</tbody>
</table>
Figure 12: The effect of heparin, DS30 and DS48 in inhibiting thrombus formation initiated by tissue thromboplastin (1 mg/kg). The data are expressed as the mean ± SEM, n ≥ 12.
Figure 13: The effect of heparin, DS30, and DS48 in inhibiting thrombus formation initiated by thrombin (12.5 U/kg). The data are expressed as the mean ± SEM, n ≥ 12.
heparin inhibited thrombus formation by ~60%. In contrast, the dose of DS30 which inhibited tissue thromboplastin-induced thrombus formation by 75% (Figure 12) was more effective in inhibiting thrombin-induced thrombus formation (p < 0.05, Figure 13). When DS48 was used, this difference was even more apparent. Thus 0.75 U/kg of DS48 was 4-fold more potent when thrombin was used as the thrombotic stimulus than when tissue thromboplastin was the thrombotic stimulus (84% and 22% inhibition, respectively, p < 0.05).

3. Factor Xa-induced thrombus formation

The relative potencies of dermatan sulfate and heparin were reversed when factor Xa was used as the thrombotic stimulus. The mean thrombus size in saline-treated animals was 96.9 ± 11.3 µl when factor Xa (70 µg/kg) was the thrombotic stimulus, consistent with the thrombus size obtained with the other thrombotic stimuli (Table 2). This value is expressed as 100.0 ± 10.2% in Figure 14 (hatched area). Ten U/kg of heparin inhibited factor Xa-induced thrombus formation by 98.4% (p < 0.001), and both DS30 and DS48 inhibited factor Xa-induced thrombus formation, but were not as effective as heparin (Figure 14).

4. Anticoagulant activity ex vivo

There was no consistent relationship between the abilities of the two batches of dermatan sulfate and heparin to inhibit thrombus formation and their anticoagulant
Figure 14: The effect of heparin, DS30 and DS48 in inhibiting thrombus formation initiated by factor Xa (20 μg/kg). The data are expressed as the mean ± SEM, n ≥ 12.
activities as measured chromogenically. The doses of dermatan sulfate which inhibited thrombus formation (75-80%) did not generate any detectable anti-factor Xa or anti-thrombin activity regardless of the stimulus used to initiate thrombus formation (Table 3). In contrast, there was a dose-related generation of anti-factor Xa and anti-thrombin activities with increasing doses of heparin (5 and 10 U/kg, respectively), measured 3 and 15 minutes post administration. Heparin (5 U/kg) generated 0.10 and 0.12 U/ml of anti-factor Xa and anti-thrombin activity respectively,  3 minutes after administration. When the dose was increased to 10 U/kg, heparin generated 0.17 and 0.19 U/ml of anti-factor Xa and anti-thrombin activity respectively (Table 3).

5. Summary

The ability of either glycosaminoglycan to inhibit thrombus formation was dependent in part on the thrombotic stimulus which induced hypercoagulation. Dermatan sulfate was more effective when thrombin was the initiating stimulus, while heparin was more effective when factor Xa was the dominating stimulus. However, dermatan sulfate was a potent inhibitor of thrombus formation regardless of the thrombotic stimulus. In addition, the antithrombotic effects of dermatan sulfate were achieved in doses which did not generate any detectable anticoagulant activity ex vivo.
Table 3: Anti-factor Xa and anti-thrombin levels before, 3 and 15 minutes after injection of heparin, DS30, DS48 or saline. Data are expressed as the mean ± SEM, n ≥ 7.

<table>
<thead>
<tr>
<th>TREATMENT</th>
<th>DOSE (U/kg)</th>
<th>ANTI-FACTOR Xa LEVELS (U/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PRE</td>
<td>3</td>
</tr>
<tr>
<td>HEPARIN</td>
<td>5.0</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td></td>
<td>10.0</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>DS30</td>
<td>2.0</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td></td>
<td>3.0</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td></td>
<td>4.0</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>DS48</td>
<td>1.0</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td></td>
<td>1.5</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td></td>
<td>3.0</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>SALINE</td>
<td>-</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>TREATMENT</th>
<th>DOSE (U/kg)</th>
<th>ANTI-THROMBIN LEVELS (U/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PRE</td>
<td>3</td>
</tr>
<tr>
<td>HEPARIN</td>
<td>5.0</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td></td>
<td>10.0</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>DS30</td>
<td>2.0</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td></td>
<td>3.0</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td></td>
<td>4.0</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>DS48</td>
<td>1.0</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td></td>
<td>1.5</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td></td>
<td>3.0</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>SALINE</td>
<td>-</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

Level of assay sensitivity: 0.05 U/ml
III. EFFECTS OF HEPARIN AND DERMATAN SULFATE ON THE TREATMENT
OF VENOUS THROMBOSIS

1. Inhibition of thrombus growth by a continuous
infusion of dermatan sulfate and heparin

Inhibition of thrombus growth by dermatan sulfate and
heparin were measured as their abilities to inhibit \(^{125}\text{I}-\)fibrin
accretion onto existing thrombi after a continuous 4 hour
infusion of either treatment. In saline-treated animals,
109.6 ± 21.8 µg of \(^{125}\text{I}-\)fibrin was accreted onto pre-formed
thrombi after 4 hours (Figure 15). When heparin was
administered in a dose of 10 U/kg/hr, fibrin accretion was not
inhibited compared to controls, 158.9 ± 37.4 µg vs 109.6 ±
21.8 µg respectively (Figure 15). When the dose of heparin
was increased to 30 U/kg/hr, fibrin accretion was
significantly inhibited by ~50% (p < 0.01). Increasing the
dose to 65 U/kg/hr had no further effect.

When DS30 was administered in a dose of 30 U/kg/hr,
fibrin accretion was inhibited by ~70%. This effect was
significantly greater than the 50% inhibitory effect obtained
with the same dose of heparin (p < 0.05). Increasing the dose
to 65 U/kg/hr had no further effect on the inhibition of
fibrin accretion. A similar effect was obtained with DS48.
A dose of 10 U/kg/hr significantly inhibited fibrin accretion
by 56% (p < 0.001). When the dose was increased to 30
U/kg/hr, fibrin accretion was inhibited by ~80%, an effect
Figure 15: The effect of heparin, DS30 and DS48 in inhibiting fibrin accretion onto existing thrombi. Doses were administered in antithrombin U/kg/hr given over 4 hours. Data are expressed as the mean ± SEM, n ≥ 14.
which was also significantly greater than that achieved with the same dose of heparin or the 10 U/kg/hr dose of DS48 (p < 0.05). Thus, dermatan sulfate inhibited fibrin accretion (thrombus growth) more effectively than heparin after a continuous infusion.

To determine a possible explanation for this difference, experiments were performed which measured the abilities of dermatan sulfate and heparin to inhibit prothrombin turnover and thrombin accretion in animals with an existing thrombus.

2. Effects of dermatan sulfate and heparin on prothrombin turnover in animals with an existing thrombus

In sham-operated, saline-treated animals, 20% of the $^{125}$I-prothrombin injected was cleared from the circulation within 10 minutes. An additional 10% was cleared in the following 20 minutes (Figure 16). There was no further clearance of circulating $^{125}$I-prothrombin over the subsequent 30 minutes (data not shown). When $^{125}$I-prothrombin was injected into saline-treated animals with an existing thrombus, $^{125}$I-prothrombin clearance was significantly increased and 50% was cleared from the circulation within the first 10 minutes (p < 0.05). There was no further clearance of the remaining $^{125}$I-prothrombin over the subsequent 50 minutes. Thus, it was concluded that i) the increase in
DOSES:
DS48: 3 ANTI-THROMBIN U/kg
HEPARIN: 10 ANTI-THROMBIN U/kg

Figure 16: The effect of a thrombus on prothrombin clearance ± DS48 or heparin. Prothrombin clearance is expressed as the percent reduction in circulating $^{125}$I-prothrombin compared to the 1 minute sample (mean, n = 5).
prothrombin consumption i.e. clearance in the first 10 minutes, reflected the interaction of circulating $^{125}$I-prothrombin with the existing thrombus; ii) this interaction resulted in the subsequent activation of $^{125}$I-prothrombin to $^{125}$I-thrombin; and iii) the $^{125}$I-thrombin was accreted onto the thrombus (the latter point will be discussed in more detail below).

When heparin was administered in a dose of 10 U/kg, 5 minutes before injecting the $^{125}$I-prothrombin, $^{125}$I-prothrombin clearance was not inhibited (Figure 16). Increasing the heparin dose 10-fold also had no effect and $^{125}$I-prothrombin clearance was similar to $^{125}$I-prothrombin clearance seen in saline-treated animals with an existing thrombus. In contrast, when DS30 was administered in a dose of 10 U/kg, the enhanced prothrombin clearance seen in animals with an existing thrombus was completely inhibited and prothrombin clearance was similar to that seen in sham-operated, saline-treated animals (Figure 16). When the dose of DS30 was reduced to 3 U/kg, $^{125}$I-prothrombin clearance was also similar to that seen in sham-operated, saline-treated animals.

The changes in $^{125}$I-prothrombin clearance in rabbits with existing thrombi ± heparin or dermatan sulfate were paralleled by corresponding changes in the amount of $^{125}$I-thrombin accreted onto the existing thrombi.
[It is assumed that the radiolabel accreted onto the thrombus in animals injected with $^{125}\text{I}$-prothrombin represents the accretion of $^{125}\text{I}$-thrombin. The rationale for this assumption is based upon the observation that there was no non-specific accretion of another plasma protein, namely albumin, onto the thrombi. Thus, in similar experiments using $^{125}\text{I}$-albumin, it was shown that i) there was no difference in clearance of $^{125}\text{I}$-albumin in sham-operated, saline-treated animals and saline-treated animals with an existing thrombus (3 ± 3 and 1 ± 1 % clearance over 30 minutes, respectively); and ii) there was no detectable accretion of $^{125}\text{I}$-albumin onto the pre-formed thrombi (Table 4).]

There was $26.4 \pm 4.0 \ \mu\text{g} \ ^{125}\text{I}$-thrombin accreted onto the existing thrombi in saline-treated animals after 30 minutes (Table 4). During this time, 50% of the $^{125}\text{I}$-prothrombin was cleared from the circulation. There was $19.1 \pm 3.0 \ \mu\text{g}$ of $^{125}\text{I}$-prothrombin accreted onto the existing thrombus in heparin-treated animals (10 U/kg). This decrease was not significant from saline-treated animals. In contrast, thrombin accretion was inhibited by 81% in DS48-treated animals (3 U/kg, $p < 0.001$).

To determine whether the effect of dermatan sulfate or heparin on prothrombin clearance and thrombin accretion were specific effects on thrombin, similar experiments were performed using hirudin, a specific thrombin inhibitor. When
Table 4 Effect of DS48, heparin and hirudin on $^{125}$I-thrombin and $^{125}$I-albumin accretion onto existing thrombi. The data are expressed as the mean ± SEM, n ≥ 10.

<table>
<thead>
<tr>
<th>TREATMENT</th>
<th>$^{125}$I-THROMBIN µg</th>
<th>%</th>
<th>$^{123}$I-ALBUMIN ND</th>
</tr>
</thead>
<tbody>
<tr>
<td>SALINE</td>
<td>26.4 ± 4.0</td>
<td>100.0</td>
<td>ND</td>
</tr>
<tr>
<td>HEPARIN (10 U/kg)</td>
<td>19.1 ± 3.0</td>
<td>72.3</td>
<td>ND</td>
</tr>
<tr>
<td>DS48 (10 U/kg)</td>
<td>5.2 ± 1.0</td>
<td>19.7</td>
<td>ND</td>
</tr>
<tr>
<td>HIRUDIN (0.1 µmol/kg)</td>
<td>7.4 ± 1.0</td>
<td>28.0</td>
<td>ND</td>
</tr>
</tbody>
</table>

ND, not detectable
animals were treated with hirudin (0.1 μmol/kg), prothrombin clearance in the presence of a thrombus was reduced to levels similar to those in sham-operated, saline-treated animals (17 ± 2% vs 18 ± 2%, respectively). Thrombin accretion onto the existing thrombus was also significantly inhibited (72%, p < 0.001) after hirudin treatment. Thus, inhibition of prothrombin consumption and thrombin accretion were best achieved using antithrombin III-independent inhibitors of thrombin, namely dermatan sulfate via heparin cofactor II or hirudin.

These results suggest that dermatan sulfate catalyses the inhibition of thrombin on an existing thrombus more effectively than heparin, since dermatan sulfate is unable to inhibit the conversion of prothrombin to thrombin (Ofosu et al, 1986). Furthermore, the ability of dermatan sulfate to inhibit both prothrombin clearance and thrombin accretion onto a thrombus was achieved when dermatan sulfate was administered as a bolus injection. These results raise the possibility therefore, that a bolus dose of dermatan sulfate is as effective as a continuous infusion, since a bolus dose of dermatan sulfate should effectively inhibit thrombin bound to a thrombus, thereby reducing the thrombogenicity of that thrombus. To test this possibility, experiments were performed to measure the relative abilities of dermatan sulfate and heparin to inhibit thrombus growth measured as
125I-fibrin accretion when both compounds were injected as a single bolus dose.

3. Inhibition of thrombus growth by a bolus injection of dermatan sulfate and heparin

There was 92.0 ± 14.0 μg of 125I-fibrin accreted onto existing thrombi in saline-treated animals after 4 hours (Figure 17). When DS48 was injected as a bolus of 3 U/kg, fibrin accretion was inhibited by 63% (34.1 ± 8.9 μg). Increasing the dose to 30 U/kg had no further effect. When heparin was injected in a dose of 3 U/kg, there was no inhibition of fibrin accretion as compared to saline-treated animals (110.8 ± 36.6 μg vs 92.0 ± 14.0 μg respectively). However, fibrin accretion was inhibited by 55% when the heparin dose was increased ~3-fold (10 U/kg, p < 0.05).

Thus dermatan sulfate and heparin inhibited fibrin accretion onto an existing thrombus more effectively when these compounds were administered as a bolus dose, rather than when given as a continuous infusion.

When DS48 was administered as a single bolus injection, the minimum dose which inhibited thrombus growth (3 U/kg) did not generate any detectable anti-thrombin or anti-factor Xa activity (Figure 18). When heparin was administered as a single bolus, the minimum dose which inhibited thrombus growth (10 U/kg) was associated with anti-thrombin and anti-factor Xa levels of ~0.12 U/ml 5 minutes
Figure 17: The effect of DS48 and heparin in inhibiting fibrin accretion over 4 hours after a bolus administration. The data are expressed as the mean ± SEM, n = 14.
Figure 18: *Ex vivo* anti-thrombin and anti-factor Xa activity over 4 hours after a single bolus injection of increasing doses of DS48. The data are expressed as the mean, SEM ≤ 0.03, n = 7.
after heparin administration (Figure 19). Increasing the bolus dose of either DS48 or heparin resulted in a dose-related increase in anti-thrombin levels 5 minutes after treatment. In heparin-treated animals, the anti-factor Xa activity paralleled the anti-thrombin activity (Figure 19), but in DS48-treated animals, there was no detectable anti-factor Xa activity (Figure 18). Thus, there was no consistent relationship between anticoagulant activity and antithrombotic effect after the bolus administration of either compound.

4. Summary

Both dermatan sulfate and heparin effectively inhibited fibrin accretion when given as a continuous 4 hour infusion at doses similar to those used clinically. However, dermatan sulfate (both DS48 and DS30) was more effective than heparin since dermatan sulfate inhibited fibrin accretion by 70-80%, whereas heparin inhibited fibrin accretion by only 50%. This difference may be explained by differences in their abilities to inhibit prothrombin clearance and subsequent thrombin accretion in the presence of an existing thrombus. Dermatan sulfate inhibited prothrombin consumption and subsequent thrombin accretion onto an existing thrombus more effectively than heparin. Dermatan sulfate, and more surprisingly heparin, also inhibited fibrin accretion onto an existing thrombus after a single bolus injection. Thus, dermatan sulfate is an effective inhibitor of both thrombin
**Figure 19:** *Ex vivo* anti-thrombin and anti-factor Xa activity over 4 hours after a single bolus injection of increasing doses of heparin. The data are expressed as the mean, SEM ≤ 0.04, n = 7.
and fibrin accretion onto an established thrombus.

IV. EFFECTS OF DERMATAN SULFATE AND HEPARIN ON tPA-INDUCED FIBRINOLYSIS

1. Possible synergistic effects of dermatan sulfate or heparin with tPA in lysing an existing thrombus

To determine whether the combination of either [tPA and heparin] or [tPA and dermatan sulfate] was more effective than tPA alone in lysing an existing thrombus, rabbits with an existing thrombus were injected with suboptimal doses of tPA, [tPA and heparin] or [tPA and dermatan sulfate].

When animals were treated with saline, thrombus size was $84.5 \pm 7.1 \mu l$ after 4 hours. This was expressed as $100.0 \pm 7.1\%$. When tPA was infused into the rabbits with existing thrombi in a dose of 30,000 IU/kg/hr (0.05 mg/kg/hr), thrombus size was reduced by $38.8 \pm 4.7\%$ ($p < 0.05$). This is expressed as a 39% increase in lysis over saline-treated animals in Figure 20. When the same dose of tPA was infused in combination with 10 or 15 U/kg/hr of heparin, the thrombolytic effect of tPA was inhibited by 50% (Figure 20, $p < 0.05$). When tPA was infused in combination with 30 U/kg/hr of heparin, the thrombolytic effect of tPA was not inhibited. However, if the dose of heparin was increased to 120 U/kg/hr, there was a trend towards an enhanced lytic effect by tPA.

When tPA was administered in combination with 3 U/kg/hr
**Figure 20:**

The thrombolytic effect of a co-administration of either DS48 or heparin with tPA, given as a 4 hour infusion. The data are expressed as the mean ± SEM, n = 16.
of DS48, there was a nonsignificant 30% enhancement of the thrombolytic effect of tPA. Interestingly, reducing the dose of DS48 2-fold (1.5 U/kg/hr) resulted in the same enhanced lytic effect as 3 U/kg/hr. When the dose of DS48 was increased to 15 and 30 U/kg/hr, this enhanced lytic effect was lost.

Thus, it appears that at suboptimal doses, DS48 tends to act synergistically with tPA, enhancing tPA-induced thrombolysis, while suboptimal doses of heparin and tPA act antagonistically preventing fibrinolysis. A possible synergistic effect between tPA and heparin was only observed when a very high dose of heparin was administered.

2. **Ex vivo anti-factor Xa and anti-thrombin levels**

The enhanced lytic effect obtained with tPA and 1.5 U/kg/hr of DS48 was achieved with no detectable anticoagulant activity *ex vivo* (Figure 21). In contrast, the enhanced fibrinolytic effect obtained with tPA and 120 U/kg/hr of heparin was achieved with marked anticoagulant activity, and steady state anti-thrombin and anti-factor Xa levels of ~1.5 U/ml were achieved (Figure 21).

Thus, both heparin and dermatan sulfate facilitated tPA-induced fibrinolysis. However, the dose of heparin required to achieve this effect was greater than the dose of dermatan sulfate. The effectiveness of the higher dose of heparin may be associated with the catalysis of thrombin
Figure 21: The *ex vivo* anticoagulant levels after a 4 hour infusion of 1.5 U/kg/hr of DS48 or 120 U/kg/hr of heparin in combination with tPA, compared to saline and tPA alone. Data are expressed as the mean, n = 9.
inhibition by heparin cofactor II. It was also determined subsequently whether this high dose of heparin is associated with a bleeding side-effect [see section V.2].

3. **Ex vivo α₂-antiplasmin levels**

The contrasting effects of heparin and dermatan sulfate on tPA-induced thrombolysis could not be explained by differential effects on circulating α₂-antiplasmin levels. In animals treated with tPA, the α₂-antiplasmin levels were 91.8 ± 17.1% after 4 hours, which were not significantly different from the α₂-antiplasmin levels in saline-treated animals after 4 hours (95.5 ± 9.0%, Figure 22). When tPA was co-administered with heparin, the α₂-antiplasmin levels were 93.9 ± 17.0%. When tPA was co-administered with DS48, the α₂-antiplasmin levels were 83.7 ± 12.2%. None of these levels were significantly different.

4. **Ex Vivo Fibrinogen Levels**

Fibrinogen levels were not significantly different in tPA-treated animals (90 ± 11% at 5 minutes and 81 ± 10% at 4 hours) compared to saline-treated animals (97 ± 8% at 5 minutes and 97 ± 7% at 4 hours). Co-administration of either DS48 and tPA or heparin and tPA also did not affect fibrinogen levels (Figure 23).

5. **Summary**

The co-administration of [dermatan sulfate and tPA] at suboptimal doses resulted in a greater reduction of
Ex vivo $\alpha_2$-antiplasmin levels after a 4 hour infusion of tPA in combination with either DS48, heparin or placebo. The data are expressed as the mean, $n = 6$. 

**Figure 22:**
Figure 23:  
Ex vivo fibrinogen levels after a 4 hour infusion of tPA in combination with either DS48, heparin or placebo. The data are expressed as the mean ± SEM, n = 6.
thrombus size than that achieved with the co-administration of suboptimal doses of [heparin and tPA]. The synergism which occurred between [DS48 and tPA] in reducing thrombus size, was achieved with no detectable anticoagulant activity ex vivo. However, synergism between [tPA and heparin] was only achieved with a dose of heparin which resulted in ~1.5 U/ml of circulating anti-factor Xa and anti-thrombin activity. The synergistic effects between either heparin or DS48, and tPA were achieved without a generalized systemic lytic state, since the α₂-antiplasmin and fibrinogen levels were unchanged throughout the 4 hour infusion.

V. BLEEDING SIDE-EFFECTS

1. Cumulative Blood Loss

The cumulative bleeding side-effects of dermatan sulfate (DS30 and DS48) and heparin were tested in a rabbit ear bleeding model over 4 hours. The doses of heparin, DS30 and DS48 used to investigate bleeding side-effects were those which maximally inhibited fibrin accretion when infused over 4 hours (30 U/kg/hr) and a 4-fold increase of this dose (120 U/kg/hr).

There was 120 ± 80 µl of blood lost over the 4 hour infusion period in saline-treated animals (Figure 24). When animals were treated with either heparin, DS48 or DS30 in a dose of 30 U/kg/hr, blood loss was not significantly enhanced
Figure 24: Cumulative blood loss over 4 hours after an infusion of either DS48, DS30 or heparin. The data are expressed as the mean, SEM = 30%, n = 6.
compared to saline-treated animals (Figure 24). When the
doses of heparin and DS48 were increased 4-fold (120 U/kg/hr),
there was also no significant increase in blood loss compared
to saline-treated animals (Figure 25).

2. Cumulative blood loss in the presence of tPA

The cumulative bleeding side-effects of a co-
administration of [DS48 and tPA] and [heparin and tPA] were
also measured, using the rabbit ear bleeding model. The same
dose of tPA that was used in the lysis experiments (30,000
IU/kg/hr) was injected alone, or in combination with a 4-fold
increase in the dose of DS48 or heparin which acted
synergistically with tPA (6 U/kg/hr for DS48 and 480 U/kg/hr
for heparin).

When animals were treated with saline, cumulative
blood loss was 169 ± 93 µl after 4 hours and reached a maximum
within 30 minutes. There was no further bleeding over the
subsequent 3.5 hours (Figure 26). When animals were treated
with tPA (30,000 IU/kg/hr), blood loss was enhanced ~4.5-fold
(800 ± 314 µl) compared to saline-treated animals, however
this enhanced blood loss was not significantly different.
When rabbits were treated with [DS48 and tPA], cumulative
blood loss was 1346 ± 570 µl over the 4 hours. Thus, enhanced
bleeding associated with the combination of [DS48 and tPA]
was not significantly different from the bleeding in either
tPA- or saline-treated animals. In contrast, when rabbits
Figure 25: Cumulative blood loss over 4 hours after an infusion of either DS48, DS30 or heparin. The data are expressed as the mean, SEM = 50%, n = 6.
Figure 26: Cumulative blood loss over 4 hours after an infusion of either tPA, [tPA and DS48] or [tPA and heparin]. The data are expressed as the mean, SEM = 25%, n = 9.
were treated with [tPA and heparin], cumulative blood loss was significantly enhanced to 17113 ± 6560 μl (p < 0.02). Furthermore, bleeding continued over the entire 4 hour period.

3. Summary

The doses of heparin and dermatan sulfate which effectively inhibited fibrin accretion after a continuous 4 hour infusion did not significantly enhance blood loss compared to saline-treated animals. However, the doses of [tPA and heparin] which acted synergistically to reduce thrombus size, significantly enhanced cumulative blood loss, whereas the combination of [tPA and DS48] which acted synergistically to reduce thrombus size, did not significantly enhance blood loss.

VI. CONTINUOUS ARTERIAL INJURY

1. Effects of dermatan sulfate and heparin on platelet and fibrin deposition in the presence of an indwelling cannula

When rabbits were injected with $^{51}$Cr-labelled platelets, killed 48 hours later, and then their ascending aorta biopsied, there was $6 \pm 6$ CPM associated per mm$^2$ of surface area. This represented 0.0001% of the radioactivity injected per ml of circulating blood, or 0.1 μl of blood contamination per biopsy sample. Consequently, "platelet deposition" to uninjured ascending aortas was considered to
be negligible, in sham-operated, saline-treated animals (Figure 27). Similar results were obtained in sham-operated animals treated with DS48 (Figure 27) or treated with heparin or aspirin (data not shown). When a cannula was inserted into the ascending aorta of saline-treated rabbits and left in situ for 48 hours, there was $5.2 \pm 1.6 \times 10^6$ platelets/mm$^2$ deposited on the aortic biopsies at the site of the cannula-induced injury (Figure 27). When the animals were treated with 30 U/kg of DS48, administered every 8 hours, platelet deposition was reduced by 21% to $4.1 \pm 0.9 \times 10^6$ platelets/mm$^2$ (Figure 27). Similar reductions in platelet deposition were obtained when the rabbits were treated with aspirin ($4.5 \pm 1.3 \times 10^6$ platelets/mm$^2$). In contrast, there was a nonsignificant 52% increase of platelet deposition onto the vessel wall at the site of injury ($7.9 \pm 2.4 \times 10^6$ platelets/mm$^2$) when the animals were treated with heparin in the same dosage regime compared to saline-treated animals. The difference between the decreased platelet deposition observed in the DS48-treated animals and the increased adhesion observed in the heparin-treated animals was significant ($p < 0.05$).

Similar results were obtained when fibrin deposition was measured on the damaged vessel wall. Fibrin deposition in sham-operated animals was negligible and also comparable to 0.1 µl of blood and therefore, was also considered to be background contamination (Figure 28). When a cannula was
The effects of DS48, heparin and aspirin on platelet deposition after 48 hours, in response to a continual arterial injury. The data are expressed as the mean ± SEM, n ≥ 9.
Figure 28: The effects of DS48, heparin and aspirin on fibrin deposition after 48 hours in response to continuous arterial injury. The data are expressed as the mean ± SEM, n ≥ 9.
inserted into the ascending aorta of saline-treated animals, 42.6 ± 8.5 μg/mm² of fibrin was deposited on the injured vessel wall (Figure 28). When animals with an indwelling cannula were treated with DS48, fibrin deposition was decreased by 18% to 34.7 ± 4.5 μg/mm². In contrast, when animals were treated with heparin, fibrin deposition was increased by 16% (49.4 ± 9.9 μg/mm²). Fibrin deposition in aspirin-treated animals was similar to fibrin deposition in heparin-treated animals. The effects of each treatment compared to saline-treated animals are summarized in Figure 29. DS48 inhibited both platelet and fibrin deposition, aspirin inhibited platelet adhesion but not fibrin deposition, and heparin enhanced both.

2. Platelet half-life

The differences in platelet deposition associated with the different treatment groups was paralleled by corresponding changes in the platelet half-life. Thus, in sham-operated animals the circulating platelet half-life was ~34 ± 2 hours (Figure 30). When a cannula was inserted into the ascending aorta and left in situ in saline-treated animals, the platelet half-life was reduced to 25 ± 2 hours. When animals were treated with DS48, the platelet half-life was 26 ± 3 hours. When animals were treated with heparin, the platelet half-life was 24 ± 2 hours, and the platelet half-life was 26 ± 2 hours in animals treated with aspirin. While these values are
Figure 29: The percent inhibition or enhancement by DS48, aspirin or heparin on platelet (A) and fibrin (B) deposition compared to saline-treated animals in response to a continuous arterial injury.
Figure 30: The effect of a continuous arterial injury on platelet half life in the presence or absence of treatment. The data are expressed as the mean ± SEM, n ≥ 9.
neither striking or significantly different, these changes in platelet half-life in the different treatment groups were significantly correlated with platelet deposition ($r = -0.969$, $p < 0.005$).

3. **Anticoagulant activity ex vivo after drug administration**

There was no detectable anti-factor Xa nor anti-thrombin activity measured 4 hours after each bolus administration of DS48, heparin or saline *ex vivo* during the 48 hour period (Figure 31).

4. **Summary**

Dermatan sulfate inhibited both fibrin and platelet deposition onto the injured vessel wall, aspirin inhibited only platelet deposition, and heparin had no effect on either platelet or fibrin deposition. There was also a significant relationship between changes in circulating platelet half-life and subsequent platelet deposition onto the vessel wall in animals treated with DS48, heparin and aspirin. Thus, DS48 appears to be a more effective inhibitor of both fibrin and platelet deposition than either aspirin or heparin.
Figure 31: Ex vivo anti-factor Xa and anti-thrombin levels measured at 4, 20, 28, 44 and 48 hours. The data are expressed as the mean, SEM $\leq 0.04$ U/ml, n $\geq 9$. 
CHAPTER 4: DISCUSSION
Heparin is the most commonly used anticoagulant both for the prevention and treatment of venous thrombosis, and as an adjunct with antiplatelet and fibrinolytic drugs in the treatment of arterial thrombosis. However, there are a number of limitations associated with the use of heparin in certain clinical situations which suggest that alternative drugs may be more effective in both the prophylaxis and treatment of thrombosis. For example, it is well established that the dose of heparin required to treat thrombosis is higher than the dose required to prevent thrombosis. This suggests that heparin is not as effective in catalysing the inhibition of activated coagulation factors by antithrombin III in the presence of an established thrombus. More recent studies demonstrate that another glycosaminoglycan, dermatan sulfate, which catalyses thrombin inhibition by heparin cofactor II, may be just as effective or more effective than heparin as an antithrombotic agent. This suggests that another endogenous coagulation inhibitor, namely heparin cofactor II, may also play an important role in achieving an antithrombotic effect. Finally, the role of heparin as an adjunct in thrombolytic therapy has received considerable attention, and while heparin appears to provide additional short-term benefits against the recocclusion of coronary arteries, the interactions between tPA and heparin in vivo are not well understood. Therefore, this
study was undertaken to gain insight into the mechanisms of action of heparin in these thrombotic situations and to determine the potential benefits of an alternative antithrombotic agent, dermatan sulfate.

I. PHARMACOKINETICS AND PHARMACODYNAMICS OF DERMATAN SULFATE

To compare the antithrombotic effects of dermatan sulfate and heparin at equivalent doses in the various thrombosis models, it was necessary to determine the pharmacokinetics of dermatan sulfate. [The pharmacokinetics of heparin have been previously determined by Boneu et al, 1987a]. The observation that the clearance of dermatan sulfate was independent of the dose administered, suggests that dermatan sulfate is cleared by a non-saturable mechanism. Thus, the most likely route of clearance is renal excretion which is similar to most low molecular weight heparins (Boneu et al, 1987b). Consistent with these conclusions, Dol et al (1988b) also found that dermatan sulfate clearance was independent of the dose administered, and that the clearance of dermatan sulfate was reduced in bi-nephrectomized rabbits.

The clearance of the anti-thrombin activity of dermatan sulfate was measured to determine if it paralleled the clearance of the radiolabelled compound. If so, the pharmacokinetics of dermatan sulfate should accurately reflect the disappearance of dermatan sulfate from the circulation. This was important to determine, since it was not possible to
measure the pharmacodynamics when dermatan sulfate was administered in low doses because of the limited sensitivity of the chromogenic assay.

It was assumed that the pharmacokinetics of dermatan sulfate at all doses accurately reflected the pharmacodynamics of the compound because, i) the pharmacodynamics of higher doses of dermatan sulfate paralleled the pharmacokinetics of dermatan sulfate at the same doses; ii) both the pharmacokinetics and the pharmacodynamics were independent of the dose administered; and iii) the pharmacokinetics of low dose dermatan sulfate paralleled the pharmacokinetics of high dose dermatan sulfate. If this assumption is correct, the administration of lower doses of dermatan sulfate (i.e. doses used in the following studies which generated undetectable plasma levels), are likely to be cleared from the circulation in the same manner.

The antithrombotic effects associated with dermatan sulfate treatment could not be attributed to any contaminant in the dermatan sulfate preparations which catalyse thrombin inhibition by antithrombin III. Dermatan sulfate has been extensively characterised in purified systems (Ofosu et al, 1985; 1986) and in addition, the administration of high doses of both batches of dermatan sulfate in rabbits generated no detectable anti-factor Xa activity in ex vivo plasma. Therefore, it was concluded that the antithrombotic effects associated with dermatan sulfate treatment in the following
studies could be attributed to the catalysis of thrombin inhibition by heparin cofactor II. [This statement should be qualified since all of the pharmacological studies were performed with batch 30 because batch 48 was not available at that time. Batch 30 has a small contaminant (<0.1%) which is able to catalyse antithrombin III activity, albeit this catalytic activity was not detectable in any of the ex vivo plasma samples at the doses tested. The subsequent antithrombotic studies were performed using both batch 30 and batch 48. Batch 48 has no contaminant able to catalyse the activity of antithrombin III as confirmed both by the manufacturer and our laboratory. Since the purification procedures for batch 30 and batch 48 were similar, it was concluded that the pharmacokinetics of batch 48 are the same as batch 30.]

If these assumptions are correct, it can be concluded that dermatan sulfate has a longer half-life than heparin when both compounds are administered in the minimum doses which prevent thrombus formation. This may explain in part, some of the differences in the effects achieved by dermatan sulfate and heparin in the inhibition of thrombus formation. However, this difference in pharmacodynamics is unlikely to contribute a significant role to the antithrombotic effect, since the expected anti-thrombin levels achieved by heparin should be at least 2-fold the expected levels of dermatan sulfate, given the design of the prevention study. Furthermore, any
differences in the effects achieved by dermatan sulfate and heparin in the prevention of fibrin accretion onto an established thrombus cannot be explained by any significant differences in their pharmacodynamics, since the clearance of dermatan sulfate at these higher doses was similar to the clearance of heparin at the same doses.

This does not exclude the possibility, however, that differences in the routes by which heparin and dermatan sulfate are cleared affect their antithrombotic potential. For example, when heparin is administered in doses used clinically (<100 U/kg), heparin is predominantly cleared by the endothelium (Boneu et al, 1987a; Barzu et al, 1985; 1984; Dawes and Pepper, 1979). In contrast, dermatan sulfate is cleared predominantly by the kidneys and does not bind to the endothelium (Dol et al, 1988b). It is possible therefore, that heparin may alter the antithrombotic effects contributed to by the vessel wall, and alter the "net" antithrombotic effect in vivo, whereas dermatan sulfate will not. Heparin has been shown to alter vessel wall permeability and hence the intravascular and extravascular exchange of plasma proteins, including those involved in coagulation (Blajchman et al, 1989). Whether these alterations have any inhibitory or enhancing effect on thrombus formation or thrombus growth is not known, but may merit further investigation.
II. ANTITHROMBOTIC EFFECTS OF DERMATAN SULFATE AND HEPARIN

1. Prevention of venous thrombosis

The observation that dermatan sulfate effectively inhibited thrombus formation by catalysing the inhibition of thrombin by heparin cofactor II, indicates that this mechanism of thrombin inhibition is as effective as the heparin catalysed inhibition of thrombin by antithrombin III. The observation that dermatan sulfate achieved these antithrombotic effects without generating anti-thrombin activity *ex vivo* as measured chromogenically, does not imply that anti-thrombin activity was not present in *ex vivo* plasma. These observations can be explained by the lower sensitivity of the chromogenic assay to heparin cofactor II-mediated thrombin inhibition as compared to antithrombin III-mediated thrombin inhibition. Thus, it was demonstrated that the *ex vivo* concentrations of heparin and dermatan sulfate which effectively inhibited thrombus formation, also enhanced the inhibition of exogenous thrombin two-fold in undiluted plasma when measured using SDS-polyacrylamide gel electrophoresis (Fernandez et al, 1987). Therefore, despite the differences in anticoagulant activity measured in *ex vivo* plasma using chromogenic assays, the abilities of heparin and dermatan sulfate to inhibit thrombin is similar using more sensitive methods, even though thrombin inhibition occurs through different pathways. These differences in the mechanism of inhibiting coagulation become more apparent when comparing the
same doses of either dermatan sulfate or heparin in the inhibition of thrombus formation initiated by different thrombotic stimuli.

The observations that the antithrombotic potencies of heparin and dermatan sulfate differ when thrombus formation is induced by different stimuli, supports the hypothesis that the antithrombotic effectiveness of these glycosaminoglycans depends upon the nature of the initial stimulus for thrombus formation. This is consistent with another study which demonstrated that heparin was a more effective inhibitor of thrombus formation when thrombosis was initiated with either factor IXa or factor Xa, than with thrombin (Gitel et al, 1977).

The minimum doses of thrombin, factor Xa and tissue thromboplastin which resulted in equivalent thrombus formation were 12.5 U/kg, 20 μg/kg and 1000 μg/kg, respectively. These doses correspond to 125 nmol/kg of thrombin and 0.5 nmol/kg of factor Xa. The molar concentration of tissue thromboplastin cannot be accurately calculated because of the heterogenous nature of this compound. However, thrombus size was similar in saline-treated animals despite differences in the potencies of the thrombotic stimuli. Therefore the differences in the antithrombotic effects of dermatan sulfate or heparin cannot be attributed to differences in the size of the thrombi formed in response to the different stimuli. A more likely explanation for these differences is that different steps in
the coagulation cascade must be inhibited, depending upon the initiating stimulus. If so, glycosaminoglycans with different mechanisms of action may be more effective in one situation than in another.

The observation that heparin effectively inhibited factor Xa-induced thrombus formation indicates that heparin is an effective catalyst of factor Xa inhibition, particularly considering the low concentrations of factor Xa required to induce thrombus formation. This is consistent with the observations by Gitel et al (1977). The observation that heparin was somewhat less effective in preventing thrombus formation initiated with tissue thromboplastin than factor Xa is not surprising, since tissue thromboplastin activates both the extrinsic and intrinsic pathway of coagulation. Tissue thromboplastin directly activates factor X through the formation of a complex with factor VII, and this same complex also activates factor IX (Zur and Nemerson, 1980). The observation that heparin was least effective in preventing thrombus formation when the stimulus was thrombin, may be explained by the larger molar concentration of thrombin required to induce thrombus formation. While heparin is an effective catalyst of thrombin inhibition (Rosenberg, 1989; Ofosu et al, 1986), it has been reported that trace amounts of thrombin which escape inhibition by heparin/antithrombin III can activate factor V and factor VIII (Ofosu et al, 1986; Yang et al, 1990). Thus, this thrombin can accelerate both
tenase and prothrombinase complex formation, resulting in further thrombin generation (Yang et al., 1990; Ofosu et al., 1987a). Thus, the effectiveness of heparin in preventing thrombus formation depends in part, on the initiating stimulus, and these results again illustrate that more heparin is required once thrombin has been formed than is required to prevent its formation.

The observation that dermatan sulfate prevented thrombus formation more effectively when thrombus formation was initiated by thrombin rather than by factor Xa or tissue thromboplastin, suggests that dermatan sulfate effectively catalyses thrombin inhibition despite the higher molar concentrations of thrombin present in vivo. This indicates that potentiation of the activity of heparin cofactor II can contribute to the effective regulation of coagulation in vivo. The observations that dermatan sulfate also effectively inhibited tissue thromboplastin and factor Xa initiated thrombus formation demonstrate that the specific inhibition of thrombin by heparin cofactor II inhibits thrombus formation, regardless of the initiating stimulus.

The contribution of phospholipid surfaces of activated platelets and other activated blood cells may also be important in the process of thrombus formation. It has been demonstrated that the addition of phospholipid to plasma in vitro, not only accelerates thrombin generation, but also protects factor Xa and thrombin from inhibition by
heparin/antithrombin III (Barrowcliffe et al, 1987; Ofosu et al, 1984b). In contrast to antithrombin III-mediated reactions, it has also been demonstrated that the ability of heparin to catalyse thrombin inhibition by heparin cofactor II, is enhanced in the presence of phospholipid (Ofosu et al, 1984b). It is not known whether the catalytic effect of dermatan sulfate on thrombin inhibition by heparin cofactor II is also enhanced in the presence of phospholipid. Therefore, it is possible that in an in vivo hypercoagulable state, the presence of phospholipid reduces the effectiveness of antithrombin III-induced inactivation of thrombin and enhances the effectiveness of heparin cofactor II-induced inactivation of thrombin. Therefore, these results suggest that thrombin is more sensitive to inhibition by heparin cofactor II than antithrombin III in certain hypercoagulable states, and that heparin cofactor II plays an important role in the in vivo regulation of thrombus formation.

2. Treatment of venous thrombosis

To prevent fibrin accretion onto an existing thrombus, i.e. to prevent thrombus growth, dermatan sulfate and heparin must catalyse the inhibition of thrombin. This may occur by either catalysing the inhibition of active thrombin bound to the thrombus (thereby preventing further thrombin generation in the blood exposed to the thrombus) and/or by catalysing the inhibition of free thrombin generated in the blood in the vicinity of the thrombus. The ability of a thrombus
(presumably with active thrombin bound to it) to activate circulating prothrombin is supported by the observation that prothrombin clearance was increased in the presence of a thrombus. This increased prothrombin clearance was paralleled by the accretion of thrombin onto the thrombus. This is illustrated diagrammatically in Figure 32. These two latter observations cannot be attributed to non-specific clearance and binding of plasma proteins, since albumin clearance was not changed in the presence of a thrombus nor was albumin accreted onto the thrombus.

A number of in vitro studies demonstrate that thrombin binds to fibrin and remains enzymatically active (Seegers et al, 1945; Liu et al, 1979; Wilner et al, 1981; Berliner and Sugawara, 1985; Hogg and Jackson, 1989; Weitz et al, 1990; Okwusidi et al, 1991). It has also been demonstrated that thrombin binds to fibrin at its anion binding exosite, which is required for fibrin(ogen) recognition. This anion binding exosite of thrombin is a positively charged region which is adjacent to, but distinct from, the active site (Wilner et al, 1981; Berliner and Sugawara, 1985; Bing et al, 1986; Fenton et al, 1988; Rydel et al, 1990). Recent evidence also indicates that thrombin may contain two anion binding exosites (Church et al, 1989). Finally it has also been recognised that heparin must bind to both antithrombin III and the anion binding exosite(s) of thrombin, in order to catalyse the inhibition of thrombin by antithrombin III (Hogg and Jackson,
**Figure 32:** Diagrammatic representation of thrombin present either free in solution (A) or bound to fibrin in a thrombus (B). Drawings A and B are enlargements of the circles present both on the thrombus and in the circulation.
1989). The observations by Hogg and Jackson (1989) and Weitz et al (1990) demonstrating that thrombin bound to fibrin was protected from inhibition by heparin/antithrombin III, raises the possibility that when thrombin is incorporated into a thrombus, the anion binding exosite becomes inaccessible to heparin. If so, thrombin should be as resistant to inhibition by heparin when it is bound to fibrin in a thrombus in vivo as it is resistant to inhibition by heparin in vitro. These in vitro observations would imply that the heparin associated prevention of thrombus growth can only be achieved by catalysing the inhibition of thrombin which is generated in the blood after being exposed to a thrombus. It would also suggest that at the doses currently used clinically, heparin does not effectively catalyse the inhibition of thrombin bound to the thrombus.

Okwusidi et al (1991) found that thrombin bound to fibrin was not resistant to inhibition by dermatan sulfate/heparin cofactor II in vitro. They proposed therefore, that dermatan sulfate must bind to a site distinct from the heparin anion binding exosite on thrombin to catalyse thrombin inhibition by heparin cofactor II. If so, then thrombin bound to fibrin in vivo, should also be inhibited by dermatan sulfate/heparin cofactor II.

The observations that dermatan sulfate, i) inhibited enhanced prothrombin clearance in the presence of an existing thrombus; ii) inhibited thrombin accretion onto an existing
thrombus; and iii) inhibited fibrin accretion onto an existing thrombus support this possibility. This is also supported by the observation that dermatan sulfate achieved these effects following a bolus administration. These results provide *in vivo* evidence which suggest that dermatan sulfate prevents thrombus growth by catalysing the inhibition of both thrombin bound to a thrombus and thrombin free in solution (Figure 33), while heparin primarily catalyses the inhibition of free thrombin in the vicinity of the thrombus (Figure 34).

The observation that heparin did not inhibit prothrombin clearance *in vivo* was surprising, since heparin can effectively delay prothrombin activation *in vitro* (Ofosu et al, 1986). However, the *in vitro* experiments were performed in the absence of fibrin, which may explain the discrepancy between these *in vitro* and *in vivo* results. The effectiveness of heparin in inhibiting fibrin accretion onto an established thrombus after a single bolus administration was also surprising, considering the inability of heparin to prevent prothrombin clearance in the presence of a thrombus and to only marginally reduce thrombin accretion onto the thrombus. The effectiveness of heparin may be due in part to the higher initial anticoagulant levels achieved *ex vivo*. The bolus doses of heparin which inhibited fibrin accretion also generated significantly higher anti-factor Xa and anti-thrombin activity. Heparin administered in doses which did not generate detectable anticoagulant activity, did not
Figure 33: Diagrammatic representation of thrombin inhibition by dermatan sulfate/heparin cofactor II when thrombin is present in solution (A) and when thrombin is bound to fibrin in a thrombus (B).
Figure 34: Diagrammatic representation of thrombin inhibition by heparin/antithrombin III when thrombin is present in solution (A) and thrombin protection from inhibition by heparin/antithrombin III when bound to fibrin (B).
inhibit fibrin accretion. While heparin did not inhibit thrombin accretion as effectively as dermatan sulfate, it did marginally inhibit thrombin accretion compared to untreated animals. Perhaps the ability to inactivate free thrombin in the vicinity of the thrombus, in addition to partially inactivating surface-bound thrombin, can sufficiently reduce the thrombogenicity of the thrombus over the 4 hour period tested. It would be interesting to determine the duration of the antithrombotic effect achieved by heparin compared to the antithrombotic effect achieved by dermatan sulfate, and this may merit further study.

The observations that dermatan sulfate achieved a better antithrombotic effect than heparin, also suggests that catalysing thrombin inhibition by heparin cofactor II is a more effective means of achieving an antithrombotic effect than by catalysing thrombin inhibition by antithrombin III in vivo. This is consistent with the in vitro studies. In addition, recent studies have demonstrated that hirudin and other specific thrombin inhibitors, which can inactivate thrombin bound to fibrin, are also effective inhibitors of thrombus growth (Markwardt, 1989; Hauptmann et al, 1990; Marki and Wallis, 1990). These studies are further supported by the observations that hirudin also inhibited prothrombin clearance, thrombin accretion and fibrin accretion as effectively as dermatan sulfate. It is concluded therefore, that antithrombin III-independent thrombin inhibitors may be
better antithrombotic agents than heparin in the prevention of thrombus growth.

3. Clinical Relevance

The resistance of thrombin bound to fibrin from inhibition by heparin/antithrombin III in vivo may also explain why more heparin is required to inhibit thrombus growth than is required to prevent its formation in the clinical setting (Gallus and Hirsh, 1976). It may also explain why more heparin is required to prevent thrombus formation after orthopedic surgery than after general surgery (Mannucci et al, 1976). It is assumed that there is more extensive tissue damage during orthopedic surgery than general surgery. If this is correct, it is likely that more thrombin is generated by the extensive release of tissue factor. If more thrombin binds to the injured vessel wall, it would also remain relatively resistant to low dose heparin therapy. Finally, it may provide a partial explanation for the recurrence of thrombosis in patients with established deep vein thrombi. Hull et al (1986) found that a number of patients receiving heparin therapy rethrombosed at a time when their APTT was thought to be prolonged appropriately, i.e. no different than the level of anticoagulation achieved in patients who did not rethrombose. They found that at the time of presentation, if patients did not receive adequate anticoagulation within the first 24 hours, the risk of rethrombosis increased even though these patients were
subsequently adequately anticoagulated. In addition, rethrombosis occurred an average of 11 days after the initiation of heparin treatment. This suggests that early in the thrombotic process, thrombin generation on the surface of the thrombus and/or the injured vessel wall must be inhibited by adequate doses of heparin in order to prevent recurrent thrombosis. If this is not achieved, then surface bound thrombin remains resistant to heparin therapy and when heparin therapy is discontinued, the active thrombin can stimulate further thrombin generation and subsequent rethrombosis.

III. EFFECTS OF DERMAFROM SULFATE AND HEPARIN ON tPA-INDUCED FIBRINOLYSIS

Heparin is administered in combination with tPA based upon the rationale that this combination will be synergistic, thereby facilitating thrombolysis and preventing further thrombotic events (Collen et al, 1989; Prins and Hirsh, 1991; Tiefenbrunn and Sobel, 1991). If this combination is synergistic, then low doses of tPA could be used in combination with low doses of heparin, thereby avoiding the unwanted bleeding side-effects associated with higher doses of each when given alone. However, synergism between these two compounds has not been observed clinically, since high doses of heparin must be co-administered with tPA, particularly in the first 24 hours, to prevent reocclusion after thrombolysis for up to one week (Hsia et al, 1990;
Bleich et al, 1990). These doses can also be associated with increased bleeding. In addition, there is little evidence that the combination results in reduced morbidity or mortality (GISSI-2, 1990).

The observations that the combination of "suboptimal" doses of both tPA and heparin did not result in any synergism in the animal studies, and in fact, thrombolysis was impaired in the presence of low dose heparin, suggest that the basis for the rationale concerning the combination treatment is not well understood. [The "suboptimal" dose of tPA used in this study resulted in an ~50% lysis and suboptimal doses of heparin or dermatan sulfate do not inhibit thrombus growth when administered alone]. In vitro studies (Andrade-Gordon and Strickland, 1986; Paques et al, 1986) demonstrate that the fibrinolytic effect of tPA is impaired in the presence of heparin. It has been proposed that heparin competes with fibrin for tPA, thereby reducing the thrombolytic effect of tPA (Andrade-Gordon and Strickland, 1986; Paques et al, 1986). However, these in vitro observations probably oversimplify the complex interactions which occur in vivo between tPA, heparin and the thrombus, since in addition to any direct effects heparin may have on tPA-induced lysis, heparin also acts as an anticoagulant, and presumably inhibits thrombus growth. These two effects of heparin in the presence of tPA were also investigated in vivo in a recent study by Agnelli et al (1990). They demonstrated that the co-administration of tPA
and heparin did not affect tPA-induced thrombolysis, however, it should be noted that the doses of both tPA and heparin were higher than those used in the present study. More importantly, they demonstrated that the dose of heparin which prevented fibrin accretion when given alone, did not prevent fibrin accretion when administered with tPA.

These *in vitro* and *in vivo* studies suggest that i) an established thrombus is thrombogenic while undergoing lysis induced by tPA; and ii) the administration of heparin with tPA reduces the functional concentration of heparin which can act on coagulation to inhibit further thrombus growth. These observations may partially explain the results obtained in the present study. The observation that the co-administration of suboptimal doses of heparin and tPA reduced tPA-induced thrombolysis, suggests that the specificity of tPA for fibrin in the presence of heparin is reduced and that heparin did not prevent fibrin accretion at these doses. Thus, these two effects could explain the antagonism observed between the co-administration of tPA and the lower dose of heparin. Therefore this present study also suggests that the thrombus remains thrombogenic during thrombolysis and that fibrin accretion can occur unchecked in the presence of tPA and low doses of heparin.

The observations that the antagonistic effects between tPA and heparin were no longer present when higher doses of heparin were administered with tPA, suggest that despite the
competition between tPA and heparin, heparin was present in sufficient concentrations to achieve an antithrombotic effect. Thus, high dose heparin inhibited free thrombin in the vicinity of the thrombus, thereby reducing fibrin accretion and allowing thrombolysis to occur more rapidly. This is consistent with a study by Rapold et al. (1989) who demonstrated, first, that elevated fibrinopeptide A levels (i.e. fibrinogen conversion into fibrin) were present in patients undergoing thrombolytic therapy, and second, that the administration of a high dose of heparin reduced these fibrinopeptide A levels to near basal levels. Therefore, it appears that the exposure of thrombin in the thrombus during thrombolytic therapy, can activate both prothrombin and fibrinogen in the vicinity of the thrombus, resulting in further fibrin accretion. High dose heparin was effective in reducing this thrombogenicity and subsequent fibrin accretion.

However, in contrast to the results obtained with heparin, suboptimal doses of dermatan sulfate were synergistic with a suboptimal dose of tPA. While in vitro studies with tPA and dermatan sulfate in the presence of fibrin have not been performed, it has been demonstrated that dermatan sulfate did not affect the tPA-induced generation of plasmin in the absence of fibrin (Andrade-Gordon and Strickland, 1986). This further supports the hypothesis that dermatan sulfate does not antagonize the thrombolytic effect of tPA, nor reduce the affinity of tPA for fibrin, but rather catalyses the
inhibition of both thrombin bound to fibrin and free thrombin by heparin cofactor II. This would also suggest that the thrombogenecity of the existing thrombus is reduced, and allow thrombolysis to occur more rapidly since it would not be counter-balanced by further fibrin accretion.

IV. **BLEEDING SIDE-EFFECTS**

The major side-effect associated with the clinical use of heparin therapy is bleeding. At present, dermatan sulfate is not widely used clinically, and therefore the extent of any bleeding side-effect is not known. However, dermatan sulfate is not associated with any significantly bleeding as compared to heparin, when measured in a rabbit ear blood loss model (Van Ryn-McKenna et al, 1989; Fernandez et al, 1986a). Consistent with these observations neither dermatan sulfate nor heparin enhanced blood loss when administered as a continual infusion in a dose which maximally inhibited fibrin accretion, or a 4-fold increase of this dose. Thus, at doses which inhibit fibrin accretion, with a 4-fold safety margin, neither compound had any marked bleeding side-effects.

However, the observation that the dose of heparin and tPA which tended to enhance thrombolysis also resulted in significant bleeding, demonstrates that the beneficial lytic effect achieved by this combination is associated with detrimental side-effects. More importantly the synergism observed between tPA and dermatan sulfate on thrombolysis
resulted in no significant bleeding.

The reasons for the enhanced blood loss associated with tPA and heparin are not entirely clear, but this effect is probably contributed to by a number of factors. Heparin-induced bleeding has been associated with the inhibition of coagulation, alterations in vessel wall permeability, and reduced platelet function, while dermatan sulfate has been shown to have little effect on these parameters (Van Ryn-McKenna et al, 1989; Buchanan et al, 1986; Blajchman et al, 1989; Fernandez et al, 1986b).

It has been demonstrated that there is a direct relationship between increased doses of heparin and enhanced blood loss, while doses of dermatan sulfate increased to the same degree as heparin only marginally enhanced blood loss (Fernandez et al, 1986a). Blajchman et al (1989) demonstrated that vessel wall permeability was altered in heparin-treated animals. Thus, there was an enhanced exchange of plasma proteins in these animals, which was also associated with enhanced blood loss. Interestingly, this enhanced permeability to plasma proteins was reduced when these same animals were treated with dermatan sulfate. Fernandez et al (1986b) demonstrated that doses of heparin which enhanced blood loss also inhibited \textit{ex vivo} platelet aggregation, while doses of dermatan sulfate which were increased to the same extent as heparin, did not enhance blood loss or inhibit platelet aggregation. The above effects of dermatan sulfate
and heparin may be related to the chemical composition of these compounds. It has been demonstrated that glycosaminoglycans such as heparin, which have a greater degree of sulfation and therefore are more negatively charged, enhance bleeding to a greater degree than glycosaminoglycans which have a lower degree of sulfation, such as dermatan sulfate (Van Ryn-McKenna et al, 1989). tPA can also contribute to enhanced bleeding by lysing fibrin in a forming hemostatic plug and by generating systemic hyperplasminemia after a continuous infusion (Colles et al, 1989).

These potential bleeding mechanisms did not enhance blood loss when either heparin or tPA was given alone in the dosage regime used in this study. However, the combination of the two resulted in significant bleeding, suggesting that the combined effects exerted by these two compounds were sufficient to exasperate bleeding in this model. The relative role of each potential bleeding mechanism may warrant further investigation.

V. EFFECTS OF DERMATAN SULFATE AND HEPARIN IN THE PREVENTION OF ARTERIAL THROMBUS GROWTH

The observation that there was significant platelet and fibrin deposition onto the aortic arch of animals with an indwelling cannula and a significant decrease in platelet half-life in these animals compared to sham-operated animals demonstrates the thrombogenicity of this injury. The role of
surface bound thrombin in the thrombogenicity of this injury is difficult to conclude from this study, however, since the results obtained after dermatan sulfate and heparin treatment were not significantly different from control animals. However, the importance of surface bound thrombin in initiating arterial thrombus growth (i.e. platelet and fibrin deposition) is consistent with other studies. Hatton et al (1989) found that thrombin bound to an injured vessel wall in vivo, was still active for up to 10 days after the initial injury. In addition, they demonstrated that thrombin bound to a subendothelial matrix in vitro enhanced fibrin accretion. Fibrin accretion onto the subendothelial matrix in vitro was also inhibited by hirudin (Hatton et al, 1990). Heras et al (1989) also demonstrated that hirudin was a more effective inhibitor of platelet and fibrin deposition after arterial injury than even very high doses of heparin.

[It must be noted that in this preliminary study, the effectiveness of the various treatment regimes in animals with an indwelling catheter was not significantly different from untreated animals with a similar injury. However, this study is relevant since there was a beneficial trend obtained with dermatan sulfate treatment compared to untreated animals, and a significant benefit with dermatan sulfate treatment compared to heparin treatment.]

The observation that platelet deposition was decreased after aspirin treatment probably reflects the decreased
platelet reactivity associated with acetylation of the cyclo-oxygenase pathway (Buchanan et al, 1982), however, aspirin had no effect on fibrin deposition. The observation that dermatan sulfate significantly inhibited both fibrin and platelet deposition compared to heparin after both were administered every eight hours as a bolus dose in equivalent anti-thrombin units, suggests that dermatan sulfate is able to inhibit surface bound thrombin more effectively than heparin. Since dermatan sulfate has little effect on platelet function at the doses used (Fernandez et al, 1987), the decreased platelet deposition in the dermatan sulfate-treated animals can best be attributed to the specific inhibition of thrombin. This would reduce the stimulus for both further platelet and fibrin deposition. Therefore, these preliminary results suggest that the catalysis of thrombin inhibition by dermatan sulfate/heparin cofactor II is an effective route for achieving an antithrombotic effect in the arterial system.
SUMMARY

This study was performed to determine whether the catalysis of thrombin inhibition by dermatan sulfate/heparin cofactor II is effective in preventing and treating thrombosis compared to the catalysis of thrombin inhibition by heparin/antithrombin III. The results of this study can be summarized as follows.

1. Dermatan sulfate clearance is independent of the dose administered, and thus is presumably cleared from plasma by a non-saturable route, most likely renal clearance.

2. Measurement of the pharmacokinetics of dermatan sulfate accurately reflects the measurement of the pharmacodynamics of dermatan sulfate.

3. Dermatan sulfate prevents thrombus formation more effectively than heparin when the stimulus for thrombus formation is thrombin. In addition, dermatan sulfate is an effective inhibitor of thrombus formation regardless of the thrombotic stimulus. In contrast, heparin most effectively prevents thrombus formation when the thrombotic stimulus is factor Xa.

4. The antithrombotic effects of dermatan sulfate are achieved by catalysing thrombin inhibition by heparin cofactor II.

5. The inhibition of thrombin bound to fibrin can be achieved by a single administration of dermatan sulfate. Since heparin/antithrombin III is less effective in
inactivating thrombin bound to fibrin, an antithrombotic effect with heparin can only be achieved with higher doses.

6. Dermatan sulfate acts synergistically with tPA, enhancing thrombolysis. The combination of derman sulfate and tPA does not enhance blood loss. Heparin also acts synergistically with tPA to enhance thrombolysis, but synergism is only achieved by doses which are associated with significant bleeding.

7. Preliminary evidence suggests that derman sulfate may reduce the thrombogenicity of an injured vessel wall, thereby decreasing fibrin and platelet deposition, and may achieve this effect more effectively than heparin.


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