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The Action of a Synthetic Pentasaccharide on Thrombin Generation in Whole Plasma

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Key words

Heparin – Pentasaccharide – Thrombin generation – Factor X_a

Summary

We investigated the effect on thrombin generation in plasma of the pentasaccharide that represent the AT III/binding site in heparin. This compound has no effect on the breakdown of thrombin in plasma. It dose-dependently inhibits the formation of thrombin in both the intrinsic and the extrinsic pathway. If coagulation is triggered by the complete prothrombinase complex (phospholipid – factor V_a – factor X_a) under conditions in which the large majority of factor X_a is bound to the complex, the inhibition of prothrombinase activity is only minor. If no factor V_a is present or if the prothrombinase activity is triggered by adding complete tenase (PL-FVIII$_a$-FIX$_a$) or incomplete tenase (PL-FIX$_a$) to the plasma the inhibition by pentasaccharide is of the same magnitude as that in the intrinsic or extrinsic system.

We conclude that the pentasaccharide inhibits blood coagulation by katalysing the inactivation of free factor X_a. In contrast to classical heparin it does inhibit the peak of thrombin formation in platelet rich plasma, probably because it is less subject to inactivation by heparin binding proteins from platelets than classical heparin is.

Introduction

From data obtained with purified factors II_a and X_a added to whole plasma it has been well documented that, with decreasing molecular mass, heparin species gradually loose their ability to act as a catalyst for antithrombin III dependent thrombin inactivation but maintain an activity in factor X_a inactivation (1–5). The synthetic pentasaccharide prepared by Choay et al. (6, 7) that represents the AT III binding site of heparin carries this tendency to an extreme in that it has no activity in the AT III-thrombin interaction but has a persistent high anti-factor X_a activity (7).

Recently we developed a method to study the generation of prothrombinase activity in plasma triggered via the extrinsic and intrinsic pathways (8). We observed that the influence of standard unfractionated heparin on the inactivation of thrombin and factor X_a generated endogenously in plasma differs considerably from its action on purified factors added to plasma. The inactivation of endogenously generated factor X_a active in prothrombinase was shown to be not or hardly enhanced by unfractionated heparin (9, 10).

It follows that the mode of action of any heparin on activated clotting factors generated in plasma cannot be foreseen from studies carried out on purified factors. This prompted us to investigate the mode of action of the synthetic pentasaccharide in plasma with our methods.

In platelet rich plasma the action of a heparin is even less predictable than in platelet poor plasma. At concentrations of up to 0.4 U/ml unfractionated heparin will *not* or hardly decrease the peak amount of thrombin formed; it will only retard the appearance of the thrombin peak (9, 11). This lack of inhibition can be attributed to neutralization of the heparin by platelet factor 4 that is released from the platelets as soon as they are activated by thrombin formed in the plasma. The increase of the lagtime of thrombin formation is due to the fact that, in the presence of heparin, inhibition occurs of the small amounts of thrombin that form during the lagphase and that are responsible for the triggering of the platelets (9, 11). Again, it seemed worthwile to investigate what the behaviour of pentasaccharide would be in this setup.

Part of the results given in this article has been presented as a plenary lecture at the XIth I.S.T.H. Congress (Brussels, 1987) (9).

Materials and Methods

All chemicals were of the highest grade commercially available.

Reptilase was obtained from Laboratoires Stago (Asnières, France), the solution was prepared according to the instructions of the manufacturer.

Soybean trypsin inhibitor (batch No. 43 F-800) was obtained from Sigma (St. Louis, USA). A 10 mg/ml solution in buffer A was used.

Staphylocoagulase was prepared as described by Hendrix et al. (12) or obtained from Laboratoires Stago (Asnières, France) and prepared according to the indications of the manufacturer.

Chromogenic substrate for thrombin was H.D-Phe-Pip-Arg-pNA. 2 HCl (S2238) from KABI, Sweden.

The phospholipid vesicle preparation contained 20% phosphatidylserine and 80% phosphatidylcholine and was prepared according to Rosing et al. (13). Kaolin was "Kaolin léger" provided by B.L.B. Laboratoires du Bois de Boulogne (Puteaux, France). Thromboplastin was prepared according to Owren and Aas (14).

Buffers: A – 0.05 M Tris-HCl, 0.1 M NaCl pH 7.35, with 0.5% ovalbumin (Sigma); B – Buffer A with 20 mM EDTA pH 7.9.

The 4th International Standard Heparin was supplied by Dr. T. W. Barrowcliffe and Pentasaccharide by the Choay Institute (15). The mean molecular weight of the heparin was given as 14,000 and its specific activity was 194 U/mg. It is assumed to contain ~30% of high affinity material, so that 1 U represents approximately 1.5 µg of high affinity heparin or 0.1 nMole. The pentasaccharide has a M. W. of 1,714.

Blood from healthy donors was collected on 0.13 M trisodium citrate; nine parts of blood to one part of citrate solution. A first and a second centrifugation were performed at $900 \times g$, at 15° C for 15 min. A third centrifugation was done at 4° C, for 1 hour at $23,000 \times g$. The platelet free plasma thus obtained was stored at −80° C. It was checked that the clotting factors and the antiproteases were in the normal range. This plasma is referred to as (platelet free) normal plasma in the text.

Defibrinated plasma was obtained by mixing an aliquot of plasma with 1 : 50 volume of a reptilase solution, letting a clot form for 10 min at 37° C and keeping the clotted plasma at 0° C for 10 min. The fibrin formed was discarded by centrifugation (10 min, $5,000 \times g$, 4° C) or by winding it on a small plastic spatula. The concentrations of factors II, V, VII, VIII, IX,

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X, XI, and XII did not significantly change by the reptilase treatment as has been shown before (8).

Platelet rich plasma was obtained by centrifugation (200 × g, 15 min) of freshly drawn citrated blood from a fasting donor. The platelet count was adjusted to 3.0×10^8/ml with homologous platelet poor plasma (centrifuged for 15 min at 10,000 × g). Plastic tubes and pipettes were used throughout so as to minimize contact activation.

Human brain thromboplastin was prepared as described in Owren and Aas (14). It was homogenized in a Potter Elvehjem homogeniser for 3 min, centrifugated at 2,000 × g for 15 min and stored at $-20°$ C in 0.1 ml aliquots. Before use it was thawed, diluted with 0.05 M Tris-HCl pH 7.35 containing 100 mM of $CaCl_2$, so as to obtain a clotting time of 90 sec when incubated with normal non defibrinated plasma under the same conditions as used for the thrombin generation experiments. The dilution required was 1:40 for the batch of thromboplastin used here and between 1:30 and 1:50 with other batches. The diluted thromboplastin was incubated at 37° C for 1 hour and then kept at room temperature. In this way it remained stable for at least 4 hours. In an independent study (16), we showed that at this concentration of thromboplastin, the reinforcement loop via factor VII dependent activation of factor IX does not play a significant role: prothrombinase activities in the absence and in the presence of factor IX were similar.

Purified proteins: Human α-thrombin was prepared according to Pletcher et al. (17), active site titrated and used for establishing a standard curve. Factors V, V_a and factor X_a were obtained as described by Lindhout et al. (18). Factor VIII was prepared according to Vehar and Davie (19) with the modifications by van Dieijen et al. (20). Factor IX was prepared according to Fujikawa et al. (21) and activated by incubation with purified factor XI_a (22).

The breakdown constants of endogenous thrombin and the thrombin generation curves were obtained as described in Hemker et al. (8), k is the overall breakdown constant, k_2 is defined as the α$_2$-macroglobulin dependent breakdown constant and k_1 is the remaining part, i. e. largely AT III dependent (8).

Experimental

The pentasaccharide at concentrations of up to 10 µg/ml had no influence on the inactivation of endogenous thrombin in normal plasma. The inactivation constant k (= $k_1 + k_2$) was 1.187 ± 0.024 (n = 10) at 5 µg/ml pentasaccharide against 1.179 ± 0.015 (n = 20) for control plasma.

When k_2, the reaction constant of thrombin and α$_2$-macroglobulin was determined it appeared that, like with standard heparin (10), it was not influenced by the presence of pentasaccharide k_2 = 0.246 ± 0.008 (n = 8, S. E. M.) compared to the control k_2 = 0.253 ± 0.005 (n = 20 S. E. M.).

In Figs. 1 and 2 the influence of pentasaccharide on thrombin generation in plasma is shown. There is an evident, dose dependent inhibition, both in the intrinsic and extrinsic systems. Because thrombin decay is not influenced by the pentasaccharide this inhibition cannot be attributed to thrombin scavenging but must be attributed to the inhibition of prothrombinase or to a decreased prothrombinase generation. Either factor X_a is scavenged or its formation is inhibited.

In the course of these experiments it appeared that pentasaccharide is adsorbed by kaolin. Therefore the usual concentration of kaolin (0.25 mg/ml) used for triggering the intrinsic system was decreased tenfold. With this amount of kaolin, contact activation appeared to be triggered to the same extend as with the higher concentration but pentasaccharide adsorption is hardly apparent anymore.

From Fig. 3 it appears that there is only a minor degree of inhibition when thrombin formation is triggered by a mixture of factors X_a and V_a and phospholipid in concentrations that ensure that the large majority of factor X_a molecules is bound in prothrombinase complex. If thrombin formation is triggered by factor X_a and phospholipid alone the inhibition is much more outspoken. At intermediate factor V_a concentrations the inhibition appeared to be dependent upon the amount of factor V_a present (results not shown). In Fig. 4 it is seen that the inhibition exerted by pentasaccharide persists when thrombin formation is triggered by the complete factor X activating complex or by factor IX_a and phospholipid but without factor $VIII_a$. If thrombin formation is triggered by factor IX_a and phospholipid there is an appreciable lagtime that is dose dependently prolonged by pentasaccharide.
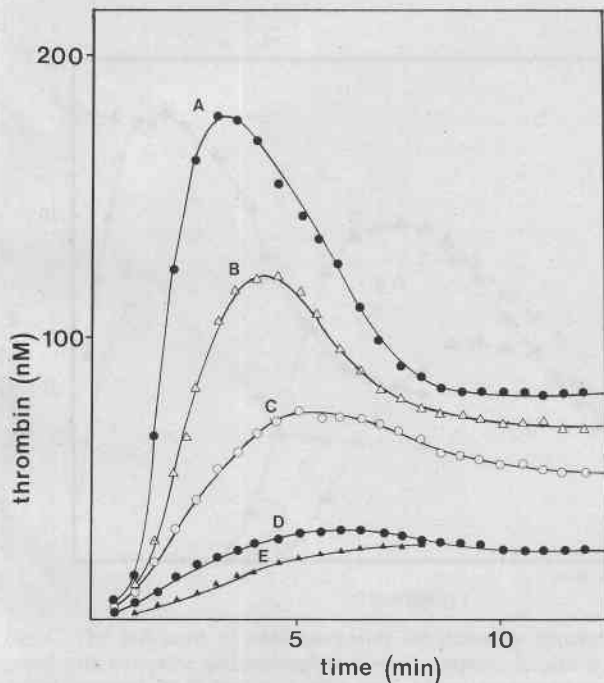

Fig. 1 The influence of pentasaccharide on thrombin generation in the extrinsic system. Reaction mixture: normal plasma 240 µl, buffer A containing heparin 60 µl, thromboplastin diluted 1:40 in $CaCl_2$ 0.1 M: 60 µl. A. Control. B. 0.05 µg/ml pentasaccharide. C. 0.1 µg/ml pentasaccharide. D. 0.3 µg/ml pentasaccharide. E. 0.6 µg/ml pentasaccharide
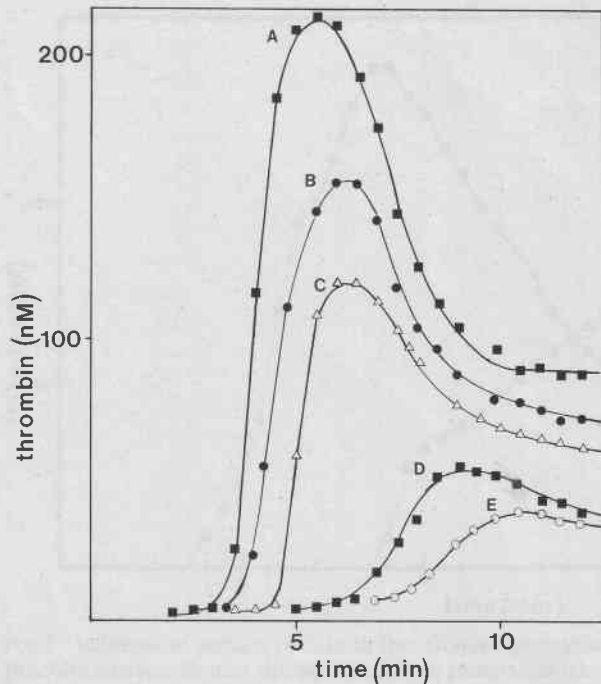

Fig. 2 The influence of pentasaccharide on thrombin generation in the intrinsic system. Reaction mixture: normal plasma 240 µl, buffer A containing: heparin, koalin (0.025 mg/ml) and phospholipid (1 µM) 60 µl, $CaCl_2$ 0.1 M: 60 µl. A. Control. B. 0.05 µg/ml pentasaccharide. C. 0.1 µg/ml pentasaccharide. D. 0.3 µg/ml pentasaccharide. E. 0.6 µg/ml pentasaccharide
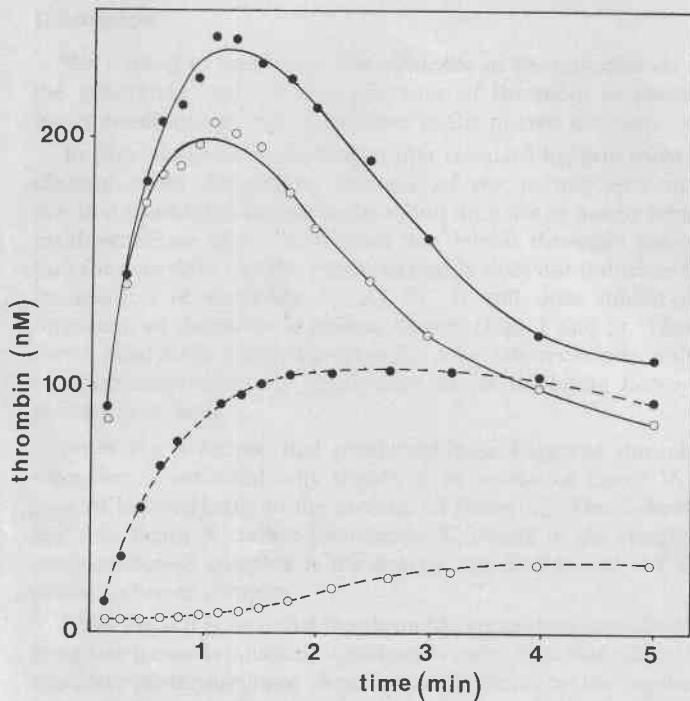
Fig. 3 The influence of pentasaccharide on thrombin formation triggered with complete and incomplete prothrombinase. Reaction mixture: 240 µl plasma, 30 µl buffer A with or without pentasaccharide, 30 µl prothrombinase, 60 µl 0.1 M CaCl$_2$. Complete prothrombinase: 5 mM CaCl$_2$, 0.1 nM factor X$_a$, 10 nM factor V$_a$, 1 µM phospholipid. Incomplete prothrombinase: 5 mM CaCl$_2$, 0.1 nM factor X$_a$, 1 µM phospholipid. ●——● complete prothrombinase; control. ○——○ complete prothrombinase; 1.2 µg/ml pentasaccharide. ●– – –● incomplete prothrombinase; control. ○– – –○ incomplete prothrombinase; 1.2 µg/ml pentasaccharide

Table 1 IC$_{50}$ values for the inhibition of prothrombinase generation by pentasaccharide and heparin in platelet free plasma

	Pentasaccharide µg/ml	Heparin µg/ml	U/ml
Intrinsic system	0.1	0.15	0.10
Extrinsic system	0.1	>0.15	>0.10
Complete prothrombinase	>1.5	>0.15	>0.10
Factor X$_a$-phospholipid	0.2	0.12	0.08
Complete tenase	0.2	0.15	0.10
Factor IX$_a$-phospholipid	0.2	<0.008	<0.005

In Table 1 we summarized the effects of pentasaccharide in terms of the concentrations that would cause 50% inhibition of the peak of prothrombinase activity in normal plasma. Because pentasaccharide does not influence thrombin breakdown its effect on prothrombinase can be read directly from its effect on thrombin generation. In Table 1 the IC$_{50}$ values of the inhibition of prothrombinase generation by heparin, obtained from the data in Béguin et al. (10), are added for comparison. The latter data are computed from thrombin generation curves and the corresponding breakdown constants.

Next we determined the effect of the pentasaccharide in platelet rich plasma. Here important differences with unfractionated heparin become apparent. Unfractionated heparin will retard the burst of thrombin formation but will not influence the peak amount of thrombin formed. The reason being that platelets, activated by trace amounts of thrombin generated during the lagtime simultaneously make available procoagulant phospholipids and heparin neutralizing platelet factor 4. Once the platelets are activated thrombin activation is explosively enhanced by the phospholipids that appear but also the heparin is virtually removed from the system (9, 11).

Pentasaccharide does prolong the lagtime, as is to be expected because it diminishes the amount of thrombin available for triggering the platelets. Contrary to unfractionated heparin it also markedly inhibits the peak of thrombin formation (Fig. 5).
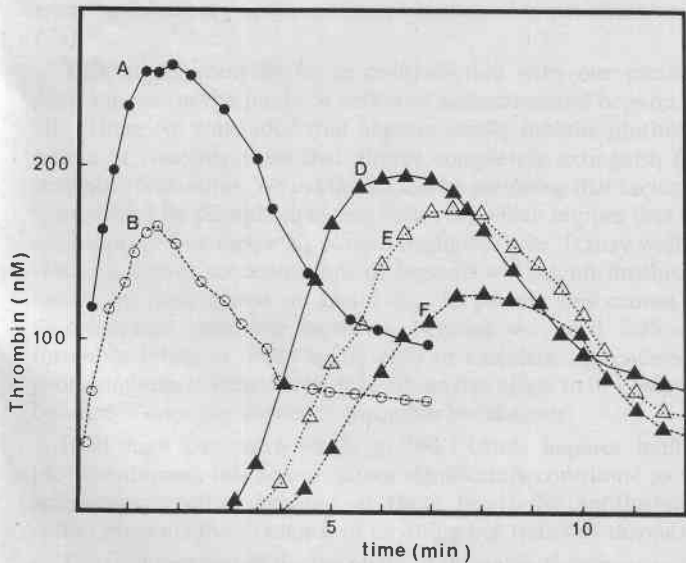

Fig. 4 The influence of pentasaccharide on thrombin generation triggered with complete and incomplete tenase complex. Reaction mixture: 240 µl plasma, 30 µl buffer A with or without pentasaccharide 30 µl tenase, 60 µl 0.1 M CaCl$_2$. Complete tenase: 5 mM CaCl$_2$, 10 nM factor IX$_a$, 5 U/ml factor VIII, 2 nM factor II$_a$, 1 µM phospholipid. Incomplete tenase: 5 mM CaCl$_2$, 10 nM factor IX$_a$, 1 µM phospholipid. A. Control: complete tenase. B. Complete tenase; 0.200 µg/ml pentasaccharide. D. Control; incomplete tenase. E. Incomplete tenase; 0.050 µg/ml pentasaccharide. F. Incomplete tenase; 0.200 µg/ml pentasaccharide
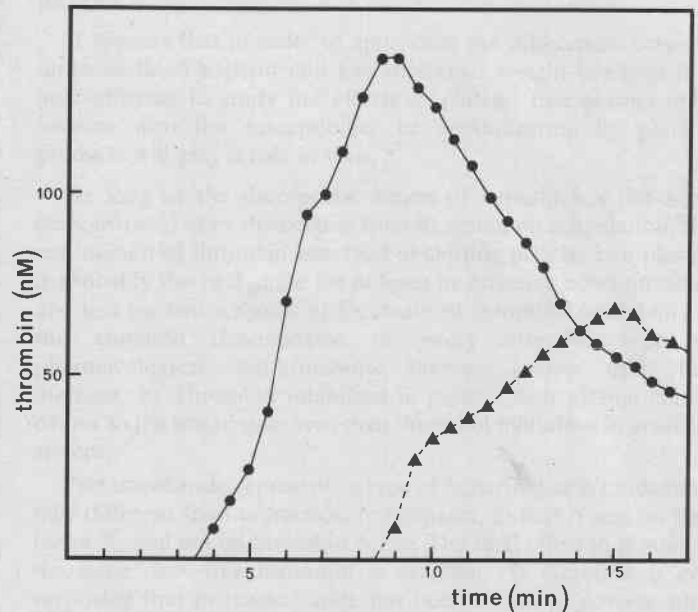

Fig. 5 Influence of pentasaccharide on the thrombin generation in PRP. Reaction mixture: Platelet rich plasma: 240 µl; pentasaccharide or buffer A: 60 µl; thromboplastin 1:400 in CaCl$_2$ 100 mM: 60 µl. ●——● control, ▲– – –▲ pentasaccharide 1.2 µg/ml

Discussion

We wanted to investigate the influence of pentasaccharide on the generation and the disappearance of thrombin in plasma, under conditions as near as possible to the in vivo situation.

In previous articles we showed that standard heparin causes a clearcut, dose dependent, increase of the pseudo first-order reaction constant of thrombin decay but does not or hardly inhibit prothrombinase at concentrations that inhibit thrombin generation for over 85% (9, 10). Pentasaccharide does not influence the inactivation of thrombin by AT III. It still does inhibit the formation of thrombin in plasma though (Figs. 1 and 2). Therefore it must act at a higher level in the coagulation cascade, either by increasing factor X_a inactivation or by inhibiting factor X activation or both.

From Fig. 3 we see that prothrombinase triggered thrombin formation is inhibited only slightly if an excess of factor V_a is present but markedly in the absence of factor V_a. This indicates that free factor X_a rather than factor X_a bound in the complete prothrombinase complex is the species attacked by the AT III-pentasaccharide complex.

From Fig. 4 it is seen that the thrombin generation provoked by complete tenase is inhibited significantly more than that caused by complete prothrombinase. Apart from the effect on the lagphase the inhibition of thrombin formation triggered by factor IX_a-phospholipid and by complete tenase (factors IX_a and $VIII_a$ and phospholipid) are comparable (Fig. 4, Table 1). This indicates that factor IX_a needs not be protected by factor $VIII_a$ probably because factor IX_a is not subject to important AT III-pentasaccharide induced inactivation.

The fact that thrombin generation induced by complete or incomplete tenase and incomplete prothrombinase is markedly inhibited whereas that induced by complete prothrombinase is not, indicates that the product of tenase, i. e. factor X_a, goes through a free stage and is not immediately bound to factor V_a and phospholipid, after its production. It also is in agreement with the observation of Barrowcliffe that pentasaccharide will hardly exert an inhibitory action on factor X_a bound in prothrombinase (23).

This might seem to be in contradiction with our previous propositions on the mode of action of unfractionated heparin (9, 10). There we concluded that heparin hardly inhibits prothrombinase at concentrations that almost completely extinguish free thrombin formation. We explained this by assuming that factor X_a is protected by phospholipid and factor V_a which implies that the inhibition of free factor X_a plays a negligible role. It may well be that still higher concentrations of heparin will inhibit prothrombinase by their action on factor X_a. In plasma this cannot be demonstrated with our methods, because we need sufficient thrombin levels in order to be able to calculate the course of prothrombinase. Pentasaccharide allows this effect to be observed because it does not influence thrombin breakdown.

If at high concentration (e. g. >0.1 U/ml) heparin inhibits prothrombinase, this action cannot significantly contribute to the anticoagulant effect because at these levels the antithrombin action prevents the existence of anything but traces of thrombin.

The prolongation of the lag phase of thrombin formation in the intrinsic system (Fig. 2) finds an unforced explanation in the fact that low ambient concentrations of thrombin during the lagphase will retard feedback activation of factor VIII. This explanation is the same as that forwarded for the prolongation of the lagphase by heparin (10), be it that the cause of the low thrombin levels during the lagtime in the case of pentasaccharide is low prothrombin activation whereas with heparin it is increased thrombin breakdown.

Heparin will inhibit intrinsic prothrombin activation to a greater extent than the extrinsic one. This is attributed to the fact that factor IX_a, in the absence of factor $VIII_a$, i. e. during the lag time, is subject to heparin-AT III dependent decay (9, 10). Pentasaccharide shows approximately the same inhibition in the intrinsic and in the extrinsic system (Table 1), again indicating that contrary to heparin, pentasaccharide does not significantly enhance the inactivation of factor IX_a in plasma. This is corroborated by the observation that it is possible to provoke notable thrombin formation by factor IX_a-phospholipid in the absence of factor $VIII_a$, whereas with heparin this is impossible (Fig. 4, ref. 10).

It has been shown that trace amounts of thromboplastin provoke the generation of traces of thrombin that will activate platelets in platelet rich plasma. The time necessary for this process is the lagtime of platelet activation. Once the platelets are activated they will make phospholipid and factor V available and explosive thrombin formation ensues. During the lagtime the system may be conceived as inactive platelets suspended in platelet poor plasma (9, 11).

Pentasaccharide, like classical heparin, will restrict the availability of trace amounts of thrombin during the lagphase, not by thrombin scavenging but by inhibition of prothrombinase formation. The prolongating effect on the lagtime is the same however. Unlike unfractionated heparin, of which up to 0.4 U/ml ($\sim$ 2.1 µg/ml) are completely neutralized by activated platelets (9, 11), pentasaccharide appears to maintain part of its activity in the presence of activated platelets because it does inhibit thrombin generation in platelet rich plasma, be it less than in platelet free plasma (Fig. 1, 2 and 5). The explanation of this phenomenon is either that the affinity of neutralizing protein from platelets is less for pentasaccharide than for heparin or that the higher molar concentration of 1.2 µg/ml of pentasaccharide ($\sim$ 0.7 µM) compared to that of 0.4 U/ml of heparin ($\sim$ 0.15 µM) represents a molar excess that exceeds the neutralizing capacity of the released proteins.

It appears that in order to appreciate the differences between unfractionated heparin and low molecular weight heparins it is not sufficient to study the effects in platelet free plasma only, because also the susceptibility to neutralization by platelet products will play a role in vivo.

As long as the therapeutic action of heparin has not been demonstrated to be dissociable from its action on coagulation, the net amount of thrombin observed in clotting platelet rich plasma is probably the best guide for judging its effective concentration, and this for two reasons: a) Decrease of thrombin availability is the common denominator of every effective type of pharmacological antithrombotic therapy known until this moment. b) Thrombin inhibition in platelet rich plasma comes nearer to the situation in vivo than thrombin inhibition in artificial systems.

Pentasaccharide represents a type of heparin that is fundamentally different from unfractionated heparin, in that it acts on free factor X_a and not on thrombin decay. The final effect in plasma is the same: less free thrombin is available. It therefore is not surprising that pentasaccharide has been shown to possess antithrombotic action in animal experiments (24).

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