The Effect of Heparin on Components of the Human Fibrinolytic System

By Kurt N. von Kaulla and Tom S. McDonald

Fibrinolysis induced in human beings for the treatment of thromboembolic disorders may remove the result of intravascular clotting, but not its underlying cause. For the prevention of the reformation of intravascular clots, a combined treatment of fibrinolysis-inducing agents with an anticoagulant is indicated.

An additional reason for combining fibrinolytic treatment with anticoagulant administration is the finding that fibrinolysis enhances clotting. This was observed in vitro by inducing fibrinolysis in human plasma with streptokinase and swine plasmin, in vivo, after injection of plasmin in dogs and during pyrogen-induced fibrinolysis in man.

Our observations of pyrogen-induced fibrinolysis in man suggest that the more recent the origin of palpable thrombi, the more rapidly and completely regression occurs. We therefore used heparin as the anticoagulant of choice during induced fibrinolysis, thus being able to start a combined treatment immediately.

On two occasions, however, it was observed that patients to whom a large dose of heparin was given shortly before the pyrogen injection did not respond in the usual manner with a marked fibrinolysis 105 to 120 minutes after pyrogen injection. These findings prompted investigation of the effect of heparin on individual components of the human fibrinolytic enzyme system. The divergent opinions on this subject expressed in the rather scanty literature seemed to us to justify these studies.

Material and Methods

It was our aim to use human components of the fibrinolytic system throughout this study. As substrate for the fibrinolytic action, however, both human and bovine material were used.

Reagents: 1. Nonfibrinolytic Materials

Plasma. Fresh citrated human plasma was used. One part sodium citrate, U.S.P. 3.8%, was mixed in the syringe with 4 parts of blood and the mixture spun for 5 minutes at 900 g.

Euglobulin fraction. Citrated plasma was diluted with 15 parts distilled water, the euglobulins precipitated by CO₂ saturation and separated by centrifugation.

Albumin fraction. Albumin was prepared from human citrated plasma in the usual way by ammonium sulfate precipitation, dialysis and lyophilization.

Antifibrinolysin. The technic of Loomis et al. was used.
Thrombin solution. Thrombin (Parke Davis) was dissolved in 50% glycerol to 200 U/cc.
Buffered saline. One part sodium barbital-acetate buffer, pH 7.42, and 4 parts 0.85% sodium chloride were mixed.
Heparin. Heparin powder (Connought) was dissolved to the calculated concentration in water. The dilution of the test enzyme solution by the added heparin solution was 0.5 to 3%.

2. Human Fibrinolytic Materials and Assays for their Activity
Urokinase (fibrinolysokinase). The activator was prepared from human urine as lyophilized powder by the method of von Kaulla.
Activated euglobulin. This was obtained by the direct action of freshly voided human urine on plasma as outlined by von Kaulla and Taylor. The material so prepared contains activated plasminogen together with urokinase.
Human fibrinolytic plasma. Marked fibrinolysis was induced by injection of purified protein-free pyrogens (Sii 1064 and 1083®). The fibrinolytic plasma was obtained 105 minutes after injection.
Activity assays. Urokinase in various amounts was dissolved in 1 cc plasma or euglobulin, 0.3 cc of the mixture was clotted with 0.01 cc. thrombin solution and the clot dissolution time measured. For these studies, urokinase amounts were chosen to produce a lysis time of 5 to 10 minutes with euglobulins, and of 20 to 90 minutes with plasma. Urine-activated euglobulins: this fraction was dissolved in a volume of buffered saline corresponding to the original plasma. Equal amounts of activated euglobulins and plasma were mixed, 0.3 cc of the mixture was clotted with 0.01 cc. thrombin solution and the clot lysis time recorded. The range was from 15 to 45 minutes. Pyrogen-activated human plasma: 0.3 cc of this plasma was clotted with 0.01 cc. thrombin and the lysis time recorded. Range: 30 to 180 minutes.

3. Substrates for Fibrinolytic Enzymes
The fibrin plate method as developed by Astrup and his group was used for most of the studies. This method makes it possible to test the activity of the fibrinolytic enzymes in the presence of anticoagulants and upon preformed fibrin. We adapted the method to our needs and developed the human plasma plate as a variation to obtain a human substrate.
Unheated bovine fibrin plate containing bovine plasminogen and thrombin. Ten cc of a 0.6% bovine fibrinogen (Armour) was dissolved in buffered saline in an Erlenmeyer flask, pH adjusted to 7.4, if necessary, and chilled. One-tenth cc. of thrombin solution was added, mixed, and the mixture rapidly poured into a carefully leveled, flat bottom Petri dish, 9 cm. in diameter, with the bottom painted black on the outside. Clotting occurred within 30 seconds. A round filter paper was glued to the inner side of the cover of the Petri dish and moistened to prevent both condensation and drying of the plate. The plate was incubated at 37 C. for 10 minutes before use.
Heated bovine fibrin plate containing neither plasminogen nor thrombin. These were prepared as indicated above, but using 0.4% bovine fibrinogen and heated at 85 C. for 45 minutes after the initial incubation.
Human plasma plates. Blood was drawn with siliconized equipment and spun at 900 g. at 4 C. for 10 minutes. Ten cc. of plasma thus obtained was either poured into siliconized Petri dishes and allowed to clot spontaneously, or 0.04 cc. of thrombin solution was added before placing the plasma in the dishes.

4. Basic Arrangement for Testing Interference of Heparin with Fibrinolysis
Euglobulin test. Heparin was added to plasma to make a final concentration of 0.2, 1, 5, 25, and 125 μg./cc. and the euglobulins then prepared. They were redissolved in the original plasma volume of buffered saline and 0.3 cc. clotted with 0.01 cc. thrombin solution. The mean lysis time of duplicates at 37 C. was recorded.
Fibrin plate method: (a) Surface measurement. Heparin was added to the various test solutions to make final concentrations of 1, 5, 25, 125, and 525 μg./cc. One standard drop (0.03 cc.) of each of the test solution-heparin mixtures was carefully placed on the surface
HEPARIN AND THE HUMAN FIBRINOLYTIC SYSTEM

of a single plate together with a control in which the heparin was replaced by buffered saline. The developing digestion areas were perfectly round if the preparation was made carefully. They were measured at intervals during incubation. The first measurement can be made as soon as the fibrin layer has been completely penetrated by the fibrinolytic enzymes, clearly exposing the black bottom of the dish. The results of the measurements were expressed in mm.\(^2\) according to the formula:

\[ \text{diameter}_1 + \text{diameter}_2 \times \pi \times \frac{2}{4} \]

is perpendicular to \text{diameter}_2.

(b) Volume determination. The volume of the lysed areas was measured in some instances by complete aspiration of the liquid contained in them by means of serologic pipets.

Results

**Heparin effect on spontaneous euglobulin lysis.** The influence of heparin on the euglobulin test was studied because this test is used by us to discover quickly, increased lysis tendency not yet detectable in the plasma.

Table 1 represents one experiment with euglobulin fractions prepared from human plasma containing increasing amounts of heparin. It can be seen that the spontaneous lysis time increased with increasing heparin content of the original plasma.

The observation of inhibition of the spontaneous lysis of euglobulins, when prepared from strongly heparinized plasma, was made in all experiments of this type. The results are open to various interpretations. Further studies on other fibrinolytic systems were done to analyze the heparin action.

**Heparin effect on urokinase-induced lysis of unheated bovine fibrin plates.** The influence of the urokinase-induced fibrinolysis on unheated bovine fibrin plates containing plasminogen was studied next. The results of a typical test of this kind are represented in figure 1. It can be seen that the growth rate of all lytic areas with all heparin concentrations and the control were practically identical. This indicates the reliability of the method. Eight experiments were performed with identical results.

Heparin at various concentrations had apparently no noticeable influence on urokinase-induced fibrinolysis by activation of bovine plasminogen.

**Heparin effect on urokinase-induced lysis of unheated bovine fibrin plates in the presence of human plasminogen.** To check what the effect may be in the presence of human plasminogen, human euglobulins were added as source of human plasminogen. Urokinase concentration in the euglobulins was 0.8 mg./

Table 1.—Spontaneous Lysis Time of Euglobulin Fractions Prepared from Plasma with Various Heparin Contents

<table>
<thead>
<tr>
<th>Heparin Concentration of the Original Plasma in µg./cc.</th>
<th>Lysis Time in Minutes</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>180</td>
</tr>
<tr>
<td>0.2</td>
<td>180</td>
</tr>
<tr>
<td>1</td>
<td>190</td>
</tr>
<tr>
<td>5</td>
<td>212</td>
</tr>
<tr>
<td>25</td>
<td>250</td>
</tr>
<tr>
<td>125</td>
<td>none in 24 hours</td>
</tr>
</tbody>
</table>
FIBRINOLYTIC SYSTEM:
UROKINASE plus BUFFERED SALINE

SUBSTRATE:
UNHEATED BOVINE FIBRIN PLATE

525 μg heparin/cc ————
125 μg heparin/cc ————
25 μg heparin/cc ————
5 μg heparin/cc ————
1 μg heparin/cc ————
control ————

cc. Control lysis time with euglobulins was 5 minutes, and with plasma, 20 minutes.

No appreciable effect of heparin on the rate of fibrinolysis could be detected. Three experiments were performed with practically identical results.

Heparin effect on lysis of unheated bovine fibrin plates induced by euglobulins prepared from human fibrinolytic pyrogen plasma. The influence of heparin on the rate and intensity of fibrinolysis could not be observed in three identical experiments in which the urokinase-euglobulin system was replaced by euglobulins prepared from highly fibrinolytic pyrogen plasma.

Heparin effect on urokinase-induced lysis of unheated bovine fibrin plates in the presence of human plasma or serum. Addition of human plasma instead of human euglobulins to the system changed the results completely. Figure 2
represents the findings in this series of experiments. Here the growth rates exhibit marked inhibition at higher heparin concentrations (525, 125 μg./cc.). At these concentrations, it was possible to perform the first measurement only after considerable delay because the fibrin layer was digested very slowly. There was an enhancement of fibrinolysis with the lower heparin concentrations. This experiment was performed 30 times with various urokinase control lysis times. Twenty-six times, inhibition was observed with two highest heparin concentrations and 11 times, enhancement with the lower concentrations. Similar results were obtained when urokinase was replaced by urine-activated euglobulins.

A clear-cut inhibition with the higher heparin concentrations was also observed in five out of five experiments when plasma was replaced by serum in the urokinase unheated bovine fibrin plate arrangement. In none of the serum experiments, however, could an enhancement be demonstrated.

**Heparin effect on urokinase-induced lysis of human plasma plates in the presence of human plasma.** When the same fibrinolytic system as shown in figure 2 (human plasma plus human urokinase), but with clotted human plasma (human plasma plate) as substrate, was used, similar results were obtained. Seven experiments of this type were performed using plasma with

![Graph](image)

**Fig. 2.—**Inhibitory and stimulatory effect of heparin on urokinase-induced fibrinolysis on unheated bovine fibrin plates in the presence of human plasma.
a platelet count of 180,000 to 250,000. In four of these, a clear inhibition with the higher concentrations (525, 125 μg./cc.) was seen, and in two of these, an enhancement with the lower concentrations (1 and 0.5 μg./cc.) was observed. In two of three additional experiments with human plasma plates in which pyrogen-plasma was substituted for urokinase-plasma, inhibition was seen with the higher heparin concentration, but no enhancement was found with the lower concentrations.

**Heparin effect on the plasmin-fibrinogen interaction.** We next tried to determine whether heparin influences fibrinolysis by interfering with the plasminogen activation, or by interfering with the plasmin-fibrinogen interaction. In the study of this problem, pyrogen plasma as a source of activated plasminogen, and heated bovine fibrin plates which contain no plasminogen or thrombin, were used.

In one typical experiment with a plasma lysis time of 132 minutes, after 680 minutes of incubation the fibrin layer was completely penetrated simultaneously by the fibrinolytic pyrogen plasma in the presence of heparin concentrations of 25, 5 and 1 μg./cc. of pyrogen plasma. At this time, fibrinolysis with the higher heparin concentrations and with the control had not advanced sufficiently to penetrate the fibrin layer. Eight experiments with pyrogen plasma and heated bovine fibrin plates were performed. In six of these, inhibition with the higher heparin concentrations was seen, and in five, enhancement with the lower concentrations was observed. We interpret these findings to mean that heparin interferes with fibrinolysis on the plasmin-fibrin level rather than with the plasminogen activation.

**Heparin effect on lysis of human plasma plates induced by human pyrogen plasma, as indicated by the volume of the digested area.** An attempt was made to measure the volume of the digested area of the fibrin plate at the end of the experiment. It was assumed that the volume would be a better indicator of the true amount of fibrin digested, since volume measurement would include the liquid released from those digestion zones in which the fibrin net was softened up and partly, but not completely, digested. Such partly digested zones may appear to the naked eye as undissolved and would therefore not be included in the surface measurement. When using volume measurement as indicator, we found, in general, more enhancement of fibrinolysis, even with the higher heparin concentrations, than with surface measurements. Figure 3 tends to confirm this. Here a pyrogen plasma was used. A plate made of the patient's own preinjection plasma served as substrate.

The corresponding surface areas were 56 mm.² for 525 μg./cc. heparin, 63 for 125 μg./cc., and 56, 56, 50, and 56 for 25, 5, 1, and 0.2 μg./cc., and 56 for the control, respectively. We concede that the volume measurement is technically difficult and wish to make the reservation that we do not as yet know how to interpret the results of volume measurements as compared with those obtained from surface area.

**Heparin effect of urokinase-induced lysis of unheated bovine fibrin plates in presence of human albumin.** As indicated by the experiments described, the presence of serum or plasma was considered to be important to bring about the heparin effect on fibrinolysis, because no such effect was demonstrable
HEPARIN AND THE HUMAN FIBRINOLYTIC SYSTEM

FIBRINOLYTIC SYSTEM: PYROGEN PLASMA
SUBSTRATE: HUMAN PLASMA PLATE

LYSIS TIME OF PYROGEN PLASMA: 50 min
INCUBATION TIME: 19 hr

VOLUME (cc) OF LYtic ZONE

<table>
<thead>
<tr>
<th>HEPARIN CONC. (µg/cc)</th>
<th>0.01</th>
<th>0.02</th>
<th>0.04</th>
</tr>
</thead>
<tbody>
<tr>
<td>525</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>125</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>25</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0.2</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>control</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Fig. 3.—Demonstration of enhancement of fibrinolysis by various heparin concentrations by means of the determination of the volume of the digested area.

with urokinase alone or with urokinase plus euglobulin. This was true also when the euglobulin fraction of pyrogen plasma was used. This discrepancy suggests that a co-factor present in serum or plasma is required for these particular heparin effects on fibrinolysis. It was found that this co-factor was contained in the albumin fraction. Figure 4 represents a typical experiment in which albumin plus urokinase was used. The albumin concentration was half of that in the plasma from which it was prepared. It is obvious from a comparison of figure 4 with figure 1 that the presence of albumin brings about an enhancement of fibrinolysis by low heparin concentrations (5 µg./cc.) and an inhibition with the higher ones (125 and 525 µg./cc.). Six comparable studies using urokinase plus antifibrinolysin were performed, but only in one could enhancement and inhibition be observed.

Table 2 gives a summary of all experiments performed. It appears that in the absence of albumin, serum or plasma heparin has no noticeable effect on fibrinolysis (Nos. 1, 2, 3). If albumin is present, inhibition with the higher heparin concentrations and, less frequently, enhancement with the lower concentrations occurs (Nos. 4-14). This effect is also present when plasminogen and thrombin-free, heated fibrin plates are used (Nos. 5, 8, 12).

DISCUSSION

The statements in the literature concerning the effect of heparin on fibrinolysis are controversial. Inhibition has been described as well as enhancement. The latter was particularly claimed to explain the regression of experimental or pathologic thrombi by heparin treatment. This regression can, however, also be observed after dicumarol or tromexan administration and is not specific for heparin. Jaques believes that heparin has no effect on fibrinolysis, or if
FIBRINOLYTIC SYSTEM:
HUMAN ALBUMIN plus UROKINASE

SUBSTRATE:
UNHEATED BOVINE FIBRIN PLATE

525 µg heparin
125 µg heparin
25 µg heparin
5 µg heparin
1 µg heparin
control

UROKINASE CONC.: 1 mg/cc. ALBUMIN SOL
CONTROL LYSIS TIME: 1 mg UROKINASE, etc.
PLASMA: 40 min.

FIG. 4.—Inhibitory and stimulatory effect of heparin on urokinase-induced fibrinolysis on unheated bovine fibrin plates in the presence of human albumin.

any effect, it would probably be to inhibit it. Heparin, and anticoagulants in general, would maintain blood flow through a thrombosed vessel by preventing further extension of the thrombus and allow the mechanism of fibrinolysis to act in this area.

The enhancement of fibrinolysis in vitro, however, cannot be explained on this basis. It depends on the thrombin amount present, according to Schmitt-Hauser-Kopp and Eichenberger. The fibrinolytic potency of thrombin would be increased many fold by small amounts of heparin which themselves are without activity. However, our experiments with heated fibrin plates showed enhancement of fibrinolysis in a thrombin-free and plasminogen system. We are therefore inclined to believe that thrombin is not a decisive factor.
HEPARIN AND THE HUMAN FIBRINOLYTIC SYSTEM

Table 2.—Summary of Experiments

<table>
<thead>
<tr>
<th>Fibrinolytic system</th>
<th>Substrate</th>
<th>Plates tested</th>
<th>Plates showing more than 10% inhibition</th>
<th>Plates showing less than 10% enhancement</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Urokinase + buff. saline</td>
<td>Unheated bovine plate</td>
<td>8</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2. Urokinase + euglobulin</td>
<td>Unheated bovine plate</td>
<td>3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>3. Pyrogen euglobulin</td>
<td>Unheated bovine plate</td>
<td>3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>4. Urine activated euglobulin + plasma</td>
<td>Unheated bovine plate</td>
<td>3</td>
<td>1</td>
<td>125</td>
</tr>
<tr>
<td>5. Urine activated euglobulin + plasma</td>
<td>Heated bovine plate</td>
<td>1</td>
<td>1</td>
<td>525, 125</td>
</tr>
<tr>
<td>6. Urokinase + serum</td>
<td>Unheated bovine plate</td>
<td>5</td>
<td>5</td>
<td>525, 125</td>
</tr>
<tr>
<td>7. Urokinase + plasma</td>
<td>Unheated bovine plate</td>
<td>30</td>
<td>26</td>
<td>525, 125</td>
</tr>
<tr>
<td>8. Urokinase + plasma</td>
<td>Heated bovine plate</td>
<td>3</td>
<td>2</td>
<td>525, 125</td>
</tr>
<tr>
<td>9. Urokinase + plasma</td>
<td>Human plasma plate</td>
<td>7</td>
<td>4</td>
<td>525, 125</td>
</tr>
<tr>
<td>10. Urokinase + plasma</td>
<td>Human plasma plate</td>
<td>5</td>
<td>1</td>
<td>125</td>
</tr>
<tr>
<td>11. Pyrogen plasma</td>
<td>Human plasma plate</td>
<td>3</td>
<td>3</td>
<td>525, 125</td>
</tr>
<tr>
<td>12. Pyrogen plasma</td>
<td>Heated bovine plate</td>
<td>8</td>
<td>6</td>
<td>525, 125</td>
</tr>
<tr>
<td>13. Pyrogen plasma</td>
<td>Human plasma plate</td>
<td>3</td>
<td>2</td>
<td>525, 125</td>
</tr>
<tr>
<td>14. Urokinase + albumin</td>
<td>Unheated bovine plate</td>
<td>9</td>
<td>8</td>
<td>525, 125</td>
</tr>
<tr>
<td>15. Urokinase + anti-fibrinolyt</td>
<td>Unheated bovine plate</td>
<td>6</td>
<td>1</td>
<td>125</td>
</tr>
</tbody>
</table>

*Optimal heparin concentration: 125, 25, 5, and 1 µg./cc.

Schmitthauer-Kopp and Eichenberger,14 Giacomazzi,4 and Vinazzer5 observed an enhancement of fibrinolysis with 1, 5, 25 µg./cc. heparin, whereas Astrup, Crookston and McIntyre2 record strong inhibition with 200–5000 µg./cc. heparin. They discuss the probability of a co-factor required for the heparin action. Most authors, dealing with the interference of heparin with spontaneous fibrinolysis, also emphasize the important fact that the enhancement is considerably reduced when heparin is added after clotting.

Our problem was to evaluate the action of heparin on fibrinolysis brought about by activation of human and bovine plasminogen induced by human activators. One type of this activation is that which the human euglobulin fraction undergoes during its preparation. We observed inhibition of the lysis of the euglobulin fraction when it was prepared from heparinized plasma. The intensity of this inhibition was roughly parallel to the heparin concentration of the original plasma. Complete inhibition was obtained only with heparin concentrations above the therapeutic level. Our results confirm the observations of Kline11 on euglobulin fractions prepared from heparinized dog blood or serum. The fibrinolytic activity induced by staphylokinase was not inhibited in their experiments.

The essential part of our study was devoted to the analysis of the interference of heparin with the lysis of preformed clots (fibrin and plasma plates); here, inhibition of fibrinolysis with high concentrations (125 and 525 µg./cc.) was a more consistent finding in all test arrangements containing albumin than was the enhancement. As indicated in table 2, all tests showing an in-
crease or decrease of the lysed area of more than 10 per cent with heparinized specimens over the control were considered as exhibiting a definite alteration. Inhibition was seen in 59 of 76 experiments (76 per cent) and enhancement in 26 (33 per cent).

It must be emphasized that in none of the 14 experiments in which no albumin was present was any enhancement or inhibition observed. For this reason we feel justified in suggesting that either albumin or the factor(s) associated with the albumin fraction is necessary for the inhibitory and stimulatory effect of heparin on the activity of human fibrinolytic components. A well founded explanation for this particular heparin action cannot be formulated at the present time. Based on the studies with the heated plates, we believe that heparin interferes with the interaction of plasmin with fibrin possibly by changing the assailability of fibrin by plasmin.

From a practical point of view, we conclude from our results that patients undergoing fibrinolysis treatment should be given a series of heparin injections with small doses rather than with few, larger, conventional ones. Thus, it would be possible to avoid any inhibition of lysis by large heparin concentrations and to profit from the enhancing effect of the small ones.

**Summary**

It was demonstrated with human urokinase, human fibrinolytic euglobulins and human pyrogen plasma as enzyme source, and with bovine fibrin plates and human plasma plates as substrate, that high heparin concentrations inhibit fibrinolysis of preformed fibrin, in contrast to small ones which may enhance this phenomenon, provided albumin is present.

No interference could be observed in the absence of plasma, serum or albumin fraction. Albumin or substances associated with the albumin fraction are required for interference of heparin with fibrinolysis. This interference probably takes place with the action of plasmin on fibrin.

**Summario in Interlingua**

Esseva demonstrate con urocinase human, euglobulinas fibrinolitic human, e plasma pyrogenic human como fonte de enzimas e con plattas de fibrina bovin e plattas de plasma human como substrato, que alte concentrationes de heparina inhibi le fibrinolyse de pre-formate fibrina, per contrasto con basse concentrationes de heparina que pote promover ille phenomeno, providite que albumina es presente.

Nulle interferentia poteva esser observate in le absentia de plasma, sero, o fraction de albumina. Albumina o substantias associate con le fraction de albumina es requirite in le inhibition del fibrinolyse per heparina. Iste inhibition occurre probablemente con le action de plasmina super fibrina.

**References**

7. ---: Antikoagulantien und Fibrinolyse. Medizinische No. 51, 1651, 1953.
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