HEPARIN EFFECT AND THROMBIN INACTIVATION

BY

M. GERENDÁS, L. PÁLOS and I. CSEFKÓ

From the Hungarian Biological Research Institute, Tihany, Lake Balaton

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In previous communications we have dealt with investigations as to the disappearance of thrombin generating in blood during clotting, respectively as to the disappearence of manufactured thrombin preparations added to the blood. We have established — on the basis of our experiments — that the disappearance of thrombin is to be attributed to two processes: a suddenly occurring adsorption and a progressive inactivation following type of a monomolecular reactions. *(Gerendás*, 1946, 1948.) In these communications we dealt in the first place with the physical and chemical laws and properties of these processes, and also with the effect of substances inhibiting inactivation.

It is known that heparin produced by the organism or administered into the blood prolongs clotting time of the blood. However till now no uniform theory has been formed on the mechanism of the heparin effect.

Some of the authors see the role of the heparin in the inhibition of the formation of thrombin (Howell and Holt 1918). According to later researches it is however beyond doubt that the disappearance of the thrombin is partly also caused by heparin (Astrup. 1943, Jorpest, 1946, Quick, 1938, and others), whereas according to Seegers and his collaborators (1942), heparin does not increase the quantity of the disappearing thrombin, but intensifies the velocity of its disappearance.

In experiments described below we have investigated what kind of connection exists between heparin and the above-mentioned adsorptive thrombin inhibition and progressive inactivating process.

METHODS

The principle of experimental determination of the thrombin inactivation consists — according to our method — in adding a solution of connection exists between heparin and the above-mentioned adthe oxalate-blood, plasma torbserum to be examined. and to observe on samples taken from it repeatedly the diminution of its activity during 10 minutes.

A standard solution from powdered thrombin is prepared, and diluted with distilled water in the proportion required for each experiment. 0,1 ml of this solution was taken and investigated in the following combination as to its clotting capacity:

Fibrinogen solution	0.1 ml.
Distilled water	0.1 ml.
Thrombin solution	0.1 ml.

The period between adding of the thrombin solution to the solution to be examined and the appearance of the first agglutinated clot which may be lifted with a glass-book, was considered as the clotting time. The experiments were carried out at room temperature.

For the preparation of the standard solution a quantity of thrombin was used, which showed in the above-mentioned solution with distilled water a clotting time of 12 seconds. This time serving as starting point corresponds to the time of activity at the beginning before the setting in of the thrombin inactivation.

To the blood, plasma or serum to be examined we added successively and in quantities detailed in the description of the experiments the activating ingredients in a glass-dish, and at last, the standard thrombin solution. After a lapse of 1, 2, 3, 5, 7, & 10 minutes of incubation time, samples were taken from the thrombin inactivating mixture and their thrombin activity in combinations similar to the above-mentioned samples was determined.

Fibrinogen solution	0.1 ml
Distilled water	0.1 „
Sample of mixture	0.1 „

The obtained clotting periods gradually lengthened indicating that the activity of the thrombin in the inactivating mixture gradually decreased as time progressed. The obtainer time-data were graphically recorded on a logarithmic scale and a straight line was drawn between them. Thus it was made possibile to interpolate the clotting times and we were able to establisch, the thrombin activity by the help of an empirical relation. From the reduction of thrombin inactivation we then calculated by the help of the formula concerning monomolecular reactions, (k=1/t. log. nat. c_0/c) the *reaction velocity constant* of the inactivation. It must be mentioned, that in order to simplify calculations a graphic method has been worked out, which will be dealt with more minutely elsewhere. (Gerendás, 1949, 1948 b.).

1. ROLE OF HEPARIN IN THROMBIN INACTIVATION

In the first place, the inactivating effect of the added heparin on thrombin inactivation was investigated through in vitro experiments. The thrombin inactivating capacity of a fresh rabbit-serum was established according to the following combination:

Serum	0.5 ml
Distilled water	0.2 „
Standard thrombin solution	0.5 „

The clotting times are shown by the normal curve of Fig. 1. The k value, calculated from the curve was: k=0.531.

It has been investigated subsequently in what proportion the velocity of inactivation changed if heparin was added to the inactivating solution.

The experiments were undertaken in the following combination:

Serum	0.5 ml
Distilled water	0.1 „
Heparin solution	0.1 „
Standard thrombin solution	0.5 „

The heparin solutions used for the mixture were chosen so that the heparine concentrations relative to the mixtures amounted to 0.3125, 0.625, 1.25, 2.50, 3.50, 5.0 and 10 μg/ml respectively.

After experiments were completed it has been established that the inactivation curves obtained by heparin concentrations of 0.3125, 0.625 and 125 μg/ml, did not differ from the normal curves obtained without the addition of heparin. After these values however the curves

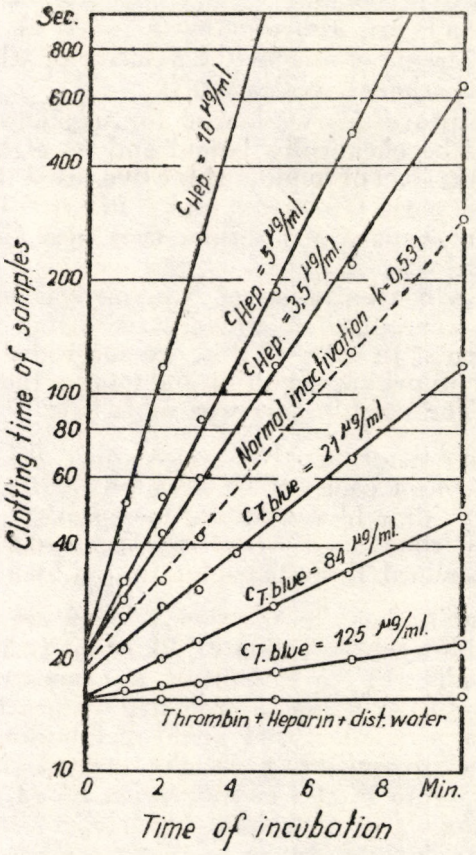

Figure No. 1. Increase of the thrombin inactivating capacity of rabbitserum on the influence of heparin, respectively its decrease on the effect of toluidine blue. The curve at the bottom shows that heparin does not produce thrombin-inactivating effect in a pure thrombin-fibrinogen system.

became steeper as the heparin concentration of the solution increased. (Fig. No. 1.). This means that with the increase of the concentration of heparin velocity of the inactivation also gradually increases. The velocity constants calculated from the data (the values of k) show a steeply rising curve. (Fig. No. 2. Heparin curve).

As the time-curves of clotting (Fig. No. 1.) start from approximately identical clotting time values, it follows that there is no change in the adsorption. Consequently it may be established, that *the effect of hepa-*

run consists in the acceleration of the reaction-velocity the process of thrombin inactivation which follows the laws of a progressive, monomolecular reaction-type.

According to our investigations the inactivation-increasing effect of heparin takes place in oxalat-blood, oxalat-plasma and also in oxalate serum obatined through cogulation by thrombin. We got similar results from the blood of man and of various animals, (ox, pig, hen, pigeon).

After these experimental results it seemed possible that the blood's own heparin contents are also playing a part in the normal inactivating process of plasma or serum (the velocity of which has been fixed in the previous experiments with $k=0.5$).

In order to prove this we looked for substances with whose help the heparin could be chemically bound and its effect thus eliminated. We investigated the effect of toluidine blue dye used for the histological demonstration of heparin *(Holmgren* and *Willander,* 1937) on the thrombin inactivation of heparinised serum. (See also Gerendás, Horn and Borsody, 1948).

Heparin in a concentration of 5 μg/ml was used for these experiments, i. e. a concentration strongly increasing inactivation in our previous experiments. In spite of this, we succeeded in finding a combination with toluidineblue, neutralising totally the effect of the heparin emplayed. The combination was as follws:

Rabbit serum	0.5 ml
Heparin solution, 60 μg/ml. 0.1	„
Toluidine blue solution, in various conc	0.1 „
Standard thrombin-solution	0.5 „

In this combination the reaction velocity amounted to $k=0.546$, with a toluidine-blue concentration of 40 μg/ml (which is expressed in a figure corresponding to the system; at a concentration of 50 μg/ml it amounted to $k=0{,}522$. From interpolated values it results that at a concentration of approximately 44 μg/ml of toluidine blue, the velocity of inactivation is corresponding to the normal inactivation, (being $k=0.531$). According to this, a *concentration of 44 μg/ml of toluidine-blue neutralizes the effect of* 6 *μg/ml heparin so that the effect of heparin in increasing inactivation is completely neutralized.*

In the subsequent experiments we investigated whether the inactivation effected by the blood itself, — in which process a part was attributed to the heparin contents of the blood — has also been influenced (decreased) by the effect of toluidine-blue.

The combination used was as follows:

Serum	0.5 ml
Distilled water	0.1 „
Toluidine blue in varying conc.	0.1 „
Standard thrombin solution	0.5 „

We employed toluidine-blue in a concentration of 0.00125, 0.0052, 0,021, 0.042, 0.081, 1,25 and 2.50 μg/ml per system, in the successive experiments.

After finishing and evaluating the experiments it has been established that velocity of inactivation was greatly reduced below the normal value by toluidine-blue. Toluidine-blue used in a very diluted solution proved effectless. Some of the curves demonstrating concentrations which diminish velocity of inactivation are shown on Fig. No. 1; the change of the reaction constant is shown by the toluidine-blue curve of Fig. No. 2.

As toluidine-blue also reduces in a large degree the inactivating capacity of the serum, it seemed beyond doubt that the heparin contents of the blood must have a part in it. Although inactivation could

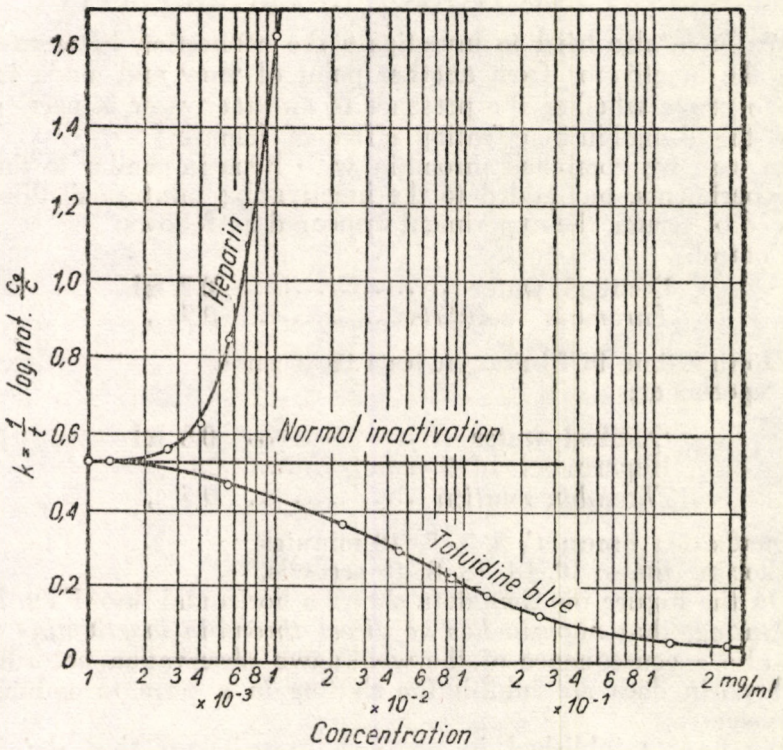

Figure No. 2. Change of value of the reactionvelocity constant of thrombin-inactivation as the effect of heparin, respectively toluidine-blue.

not be stopped entirely by toluidine-blue, the remaining slight inactivating effect was attributed to heparin liberated from the heparin — toluidineblue compound by dissociation. Thus it may be established that *the fermentative thrombin inactivation does not occur without heparin or only on a very small scale.*

In connection with experiments on other subjects we also made the observation that in adding histamine to a violet coloured heparin-toluidine blue solution the original blue colour of the toluidine-blue reappears; consequently, histamine is also bound by heparine, the bond between histamin and toluidine-blue being even stronger than that

between histamin and heparin. (Gerendás, Csefkó and Udvardy, 1948 a). Based up on this observation the effect of histamin has been examined on normal serum and on serum treated with heparin, and an effect similar to that of the toluidine-blue has been observed. (Csefkó, Gerendás und Udvardy 1948, a) These experiments also prove that the fermentative inactivator does not work without heparin. It seems possible, that also the reciprocal heparin-kinase effect — investigated by *Fischer* and *Astrup*, (1935) Horn and Borsody (1948) — manifests itself in heparin being bound by kinase diminishing thus the effect of heparin on inactivation. (Csefkó, Gerendás, Udvardy, 1948).

2. ROLE OF INACTIVATOR IN HEPARIN-EFFECT

We have also tried to investigate the connection between heparin and the inactivator from another point of view and made investigations to prove whether the presence of an inactivator is necessary to produce the thrombin-inactivating effect of heparin?

In case we combined thrombin with heparin similar to the previous experiments, but added to the inactivating mixture distilled water instead of serum, the experiment appeared as follows:

Control:

Distilled water	0.7 ml
Thrombin solution	*0.5* „

Clotting time in fibrinogen tests 18 seconds.

Experiment:

Distilled water	0.6 ml
Heparin sol. of 5 μg/ml.	0.1 „
Thrombin solution	*0.5* „

Incubating times: 1', 2, 3, 5, 10 minutes.

Clotting times: 19, 18, 18, 8, 18 seconds.

On the Figure the time data shows a horizontal line (Fig. No. 1) demonstrating, that *heparin has no direct thrombin inactivating effect.* This is also a consequence of the wellknown observation according to which heparin does not inhibit the clotting in a pure thrombin-fibrinogen system.

We have established in previous experiments that the inactivating ferment is thermolabile, and loses its entire activity when heated to 60° C (Gerendás, 1948). We now tried to determine, whether heparin had an inactivating effect on such heated plasma.

We kept ox serum for 10 minutes at a temperature of 60° C, cooled it subsequently to room-temperature, and undertook inactivating experiments in the following combination:

Heated, cooled and filtered serum	0.3 ml
Heparin solution, 100 μg/ml	0.1 „
Distilled water	0.1 „
Standard thrombin solution	0.3 „

Incubating time: 1, 2, 5, 10 minutes.

Clotting time of test: 24, 24, 25, 24 seconds.

This experiment proves that heparin does not develop its inactivating effect, if the thermolabile inactivating substance the plasma has been destroyed by heating.

DISCUSSION

The experiments described are proving *that heparin only exercies thrombin inactivating effect, respectively effect of increasing inactivation when inactivating ferment of the plasma is able to work.*

It now follows from our experiments that the inactivating ferment discussed in detail in our above-cited publications — and heparin are closely connected with one another, and that the inactivator is needing heparin for its action, just as heparin cannot exercise its inactivating effect without the presence of the inactivator.

It consequently follows that the delay of thrombin-clotting in the plasma treated with heparin is due to the fact that thrombin has been inactivated during the clotting by the influence of heparin added to the plasma, and only fractions of it remained to take part in the clotting.

Howell and *Holt* have already demonstrated (1918), that heparine did not impede coagulation in a pure thrombin-fibrinogen system. According to them a plasma-factor is necessary for producing the clotting inhibition effect of heparin. This problem since the above-mentioned statement of Howell and Holt has had an extensive literature (Jorpes, 1946) yet without a uniform opinion having been reached by the authors.

In the course of our researches we have formed the opinion that inactivation of thrombin is effected by a thermolabile, fermentlike substance, equally present in the plasma and in the serum. The activity of this substance is increased as the effect of heparin and decreased as the effect of heparin-binding substances (toluidine-blue, histamine, kinase). From this we may conclude, that the thermolabile substance corresponds to the protein part of the thrombin-inactivating ferment, whereas heparin might be regarded as the prosthetic group of this ferment, or possibly as its activator.

After the conclusion of the experimental series as above, one of our collaborators *(Pálos,* 1949) observed, that heparin is protecting thrombin against oxydative- and heat inactivation. This protective effect of heparin is in an appearent contrast to our previous experimental results. It must however, be taken into consideration, that the results of Pálos were obtained in a pure heparin-thrombin system, whereas our experiments were performed in serum. This is possibly the explication of the effect of heparin accelerating inactivation of thrombin in presence of the thermolabile factor, whereas it seems to protect thrombin against inactivation in the absence of this factor.

In physiological conditions, of course, only the previous action is possible.

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ДЕЙСТВИЕ ГЕПАРИНА И ИНАКТИВАЦИЙ ТРОМБИНА

Автор: МХАЙЛ ГЕРЕНДАШ

РЕЗЮМЕ

Опыты in vitro над способностью гепарина к инактивации тромбина и к предотвращению свертывания показали, что последний не влияет на адсорптивное исчезновение тромбина, но увеличивает в значительной степени быстроту инактивации тромбина. (прогрессивная реакция первой степени).

Для осуществления инактивации тромбина в крови требуется совместное действие двух факторов: Один из этих факторов термонабильное вещество встречающееся в равной степени в сыворотке и в плазме, другой фактор — гепарин.

Ни тот, ни другой фактор сам по себе не может инактивировать тромбин без содействия со стороны другого фактора.

Мы считаем, что термолабильное вещество соответствует белковой составной части фермента, а в тоже время гепарин соответствует предстательной группе фрегмента.