OBSERVATIONS ON THROMBIN INACTIVATION BY HUMAN SERUM*

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Early investigators of the coagulation of blood observed that the clotting principle which is formed during clotting disappears shortly after the clotting is accomplished (1, 2). Blood thus possesses a mechanism not only to produce thrombin, but also to dispose of this substance after its role has been fulfilled. The accumulation of a factor dangerous to the organism is prevented in this way. The nature of the antithrombic mechanism has been the subject of numerous investigations,[1] but it is still far from being fully understood. Astrup and Darling (4, 5) demonstrated that the antithrombic potency of plasma is bound to two distinct factors: (1) the normal antithrombin, and (2) the thrombin coinhibitor activated by heparin to thrombin inhibitor. According to these authors the normal antithrombin is not affected by heparin in any way. In the meantime Seegers and his associates (6–8) and Quick and Favre-Gilly (9) called attention to the fact that large amounts of thrombin are bound to the fibrin gel through an adsorptive process. Seegers (10) later demonstrated the formation of a new antithrombic factor during the transformation of prothrombin into thrombin in a plasma in which all the antithrombic activity had been destroyed previously by shaking with ether. None of these antithrombic factors has been isolated in a pure form; nor has their mechanism of action been completely elucidated.

It should be mentioned that with these reactions pronounced differences may be encountered with sera of different species. For example, according to Howell (11) and Quick (12) in dog, cat, and rabbit sera, heparin greatly enhances the antithrombic potency, whereas Astrup and Darling (5) were unable to increase the antithrombic effect of bovine serum with heparin. Udvardy (13) described differences also in the type of thrombin inactivation curves and in the rate of the inactivation with sera of different mammals.

In the present paper investigations will be described on the thrombin-inacti-

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[1] For detailed bibliography see Volkert's monograph (3).

vating capacity of human serum. Serum has been employed rather than plasma, in order to avoid the adsorptive inactivation, dealing thus only with the true antithrombic components of serum. The emphasis has been laid on the rate of the disappearance rather than on the amount of thrombin inactivated, most of the experiments being conducted with relatively small amounts of thrombin.

Methods

Human blood of healthy subjects was obtained by venipuncture with sterile dry syringes and then transferred to centrifuge tubes. The clot formed was gently pressed with a glass rod and the serum centrifuged, then left for an hour at room temperature for the completion of the consumption of prothrombin (9) and inactivation of thrombin. Serum not older than 24 hours was used in the experiments, except in the case in which the effect of storage upon the thrombin-inactivating factor was investigated.

Preparation of Thrombin.—For the experiments related in this paper, bovine prothrombin was prepared according to Seegers (14). Prothrombin was converted into thrombin either with human brain thromboplastin in the presence of calcium, or with sodium citrate as described by Seegers *et al.* (15). The purity of the product was of 150 to 300 NIH[2] units per mg. of protein. There was no change in the activity of the concentrated solutions during a period of 1 month's storage in the refrigerator.

Fibrinogen.—Bovine fraction I, from Armour Laboratories, lot No. 128–163, was used without further purification.

Commercial preparations of heparin sodium from Abbott Laboratories, protamine sulfate from Eli Lilly and Co., and crystalline soy bean antitrypsin from the Worthington Laboratories were employed. Toluidine blue 0, of 66 per cent dye content, was obtained from Coleman and Bell Company. All the other chemicals were of reagent grade.

Clotting Time Determination.—Clotting times were determined with a method originally described by Bürker (16) and modified later by Laki (17). Portions of 0.2 ml. each, of fibrinogen solution are pipetted into the depressions of a porcelain spot plate. From a serological pipette 0.2 ml. of the solution to be tested is blown into the fibrinogen solution, and effective mixing is achieved by blowing a current of air into the mixture. A stop-watch is started at the same time. At regular time intervals a small glass hook is immersed in the solution, and clotting time is considered to be the time elapsed until the first voluminous thread can be withdrawn with the hook. It was found that the most satisfactory results, with regard to the sharpness of the end-point and linearity of the plot of clotting time *vs.* thrombin dilution, were obtained with a 2 per cent bovine fraction I solution in 0.2 M NaCl, 0.05 M sodium phosphate buffer of pH 7.23. With the above method 1.5 NIH thrombin units in 0.2 ml. of solution give a clotting time of approximately 20 seconds at 25 ± 1°C. This clotting time was obtained consistently with freshly prepared fibrinogen solutions, but the clotting time prolonged with the aging of the solution. However, the fibrinogen solutions did not change their clotting ability in a period of 1

[2] National Institutes of Health.

week, if kept in the frozen state at $-15°C.$, and incubated before use in a water bath of 36°C. for 10 minutes.

Thrombin Inactivation Test.—The method of Gerendás (18) was modified slightly. In a pyrex test tube 0.8 ml. of serum was mixed with 0.2 ml. of 0.5 M phosphate buffer of pH 7.23 and sufficient saline solution to give after the thrombin addition a final volume of 1.5 ml. The tube was placed in a constant temperature water bath at 24°C. and, after reaching the temperature equilibrium, thrombin solution of the same temperature was added. At time intervals samples of 0.2 ml. were withdrawn and their clotting ability determined. The amount of thrombin used in these experiments varied between 30 and 40 units in 1.5 ml. of reaction mixture, which gave a clotting time of about 10 seconds with normal serum after an incubation of 30 seconds. The decrease of the thrombin activity was usually followed for 6 minutes.

Some experiments were conducted with thrombin concentrations ranging from 300 to 600 units in the reaction mixture. 1 ml. of serum was mixed with 0.2 ml. of 0.5 M phosphate buffer of pH 7.23 and 1.0 ml. of thrombin solution, and the mixture was incubated at 24 or 38°C. The samples of 0.2 ml. withdrawn at given time intervals were diluted 10 to 20 times before testing their thrombic activity. In some instances the 0.2 ml. samples were blown from a serological pipette into 4.8 ml. of fibrinogen solution, the dilution being effected in this way with the fibrinogen solution itself, and the clotting time determined with the Klett-Summerson photoelectric colorimeter as described by Laki (17).

RESULTS

Kinetics of the Inactivation of Thrombin.—The clotting time is inversely proportional to the thrombin concentration (19), therefore:

$$\log C_t = \log a - \log (Th)$$

in which (Th) is the thrombin concentration, a a proportionality constant, and C_t the clotting time. If the disappearance of thrombin follows a first order kinetics, log (Th) plotted against reaction time should result in a straight line, the negative slope of which is the rate constant, k:

$$\log (Th) = \log (Th_0) - kt$$

As can be seen from the above equation, a plot of log C_t *vs.* reaction time should result also in a straight line, its slope equal to k. Fig. 1 shows that with low thrombin concentration and different serum concentrations this relationship is fulfilled, the reaction being thus of an apparent first order. Glazko and Ferguson (20), and Gerendás (18, 21) arrived at similar conclusions. In all the experiments performed with thrombin concentrations of the order of 40 units per ml. of serum and phosphate buffer of pH 7.23 the first order kinetics was strictly obeyed. Therefore, subsequently the rate of the thrombin inactivation will be expressed by the first order rate constant calculated with decimal logarithms, with time expressed in minutes.

Increasing the thrombin concentration modifies the reaction type. With

thrombin concentrations of 300 to 600 units per 2.2 ml. of reaction mixture the reaction proceeds much more slowly, as is shown in Fig. 2, and not the logarithm, but the clotting time itself increases linearly with the incubation time. The slope of the straight lines decreases with increasing thrombin concentration.

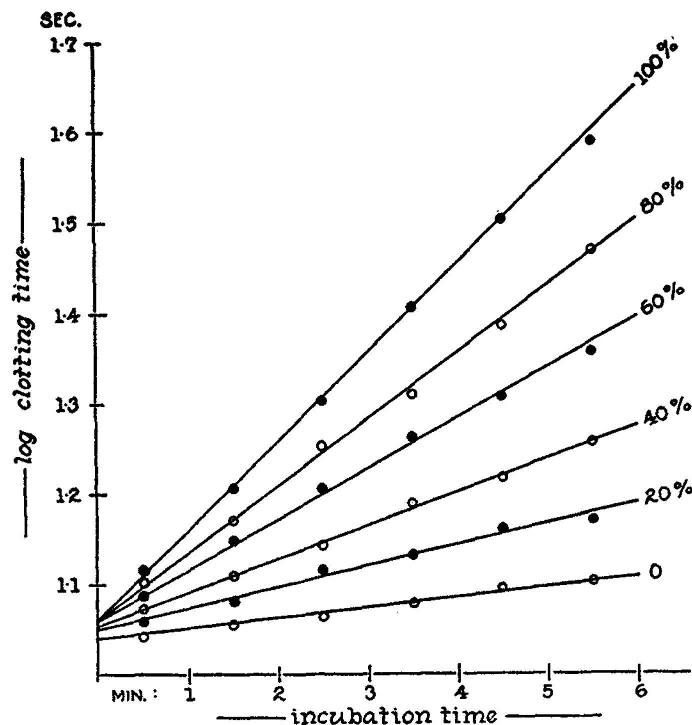

FIG. 1. Effect of serum concentration on the rate of inactivation of thrombin with 40 units of thrombin in the reaction mixture. The lines correspond to 0, 20, 40, 60, 80, 100 per cent serum concentration. Ordinate, log of clotting time; abscissa, incubation time.

There was no evidence of an immediate disappearance of thrombin at the moment of mixing with serum, as claimed by Gerendás (18, 21). The intercept of the lines of Fig. 1 with the ordinate corresponded exactly to the logarithm of the clotting time of a blank prepared in a paraffin-coated tube with physiological saline instead of serum.

To a series of test tubes each containing 0.8 ml. of serum and 0.2 ml. of 05 M phosphate buffer of pH 7.23, varying quantities of thrombin, from 25 to 100 units, were added and the volume made up to 1.4 ml. with saline. The mixtures

were incubated at 37°C. for an hour. At the end of this period practically no free thrombin was left in the solutions. The mixtures were then placed in the 24°C. water bath, and adding 30 units of thrombin the rate of the disappearance of thrombin was determined. As can be seen in Fig. 3, the rate decreased linearly in the initial portion of the curve, with the amount of thrombin inactivated in the first phase of the experiment. This experiment demonstrates that the antithrombic potency of serum is limited and can be exhausted.

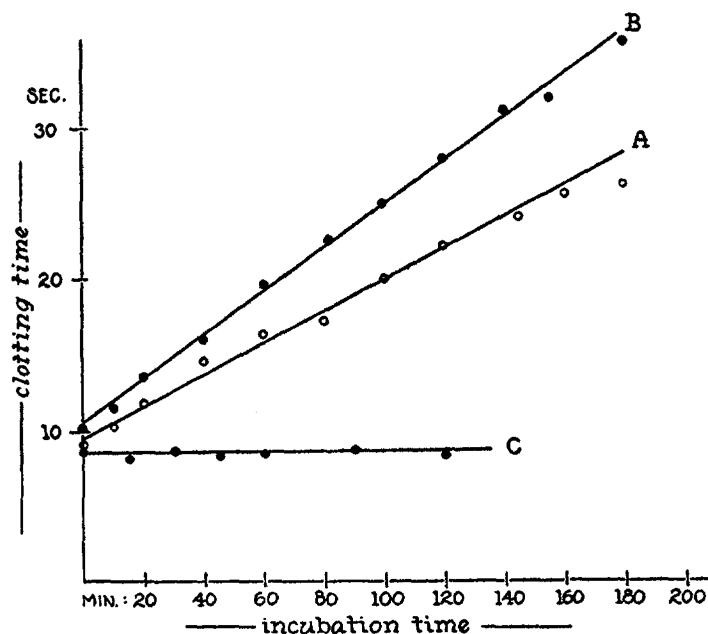

FIG. 2. Thrombin inactivation with 300 units of thrombin per ml. of serum. Curve A, serum alone; curve B, serum with 0.06 mg. of heparin per ml.; curve C, blank with physiological saline.

Serum inactivates thrombin at a lower rate than plasma. This difference is explained readily on the basis of the preceding experiment. Namely, in serum there will be less antithrombin available for the exogenous thrombin than in plasma, because some of the antithrombic potency has, already been used to neutralize the thrombin generated during clotting. With small amounts of thrombin as substrate, Wilson (22–24) found no difference between the antithrombic effect of serum and plasma. However, his experiments with very dilute systems are likely to give erroneous results due to the pronounced spontaneous inactivation of thrombin. Later, Owen and Bollman (25) reported significantly lower antithrombin values with serum than with plasma. Seegers,

Miller, Andres, and Murphy (26) could demonstrate that in equilibrium conditions, with high initial thrombin concentration, 1 ml. of serum inactivates approximately 230 units of thrombin less than the same amount of plasma; *i.e.*, a difference equal to the amount of thrombin generated and then inactivated during the clotting of blood.

Stability of the Antithrombic Factor.—The rate of the thrombin inactivation was determined with serum 2 hours after obtaining the blood and the determination was repeated daily for a period of 14 days. In this period of time the

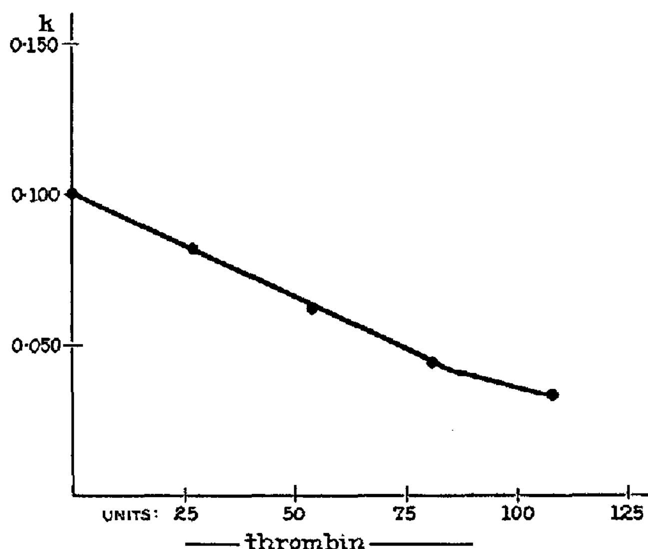

FIG. 3. Exhaustion of the antithrombic potency. The amounts of thrombin shown on the abscissa were added to serum samples and after complete inactivation of these, the remaining activity was tested on a second thrombin addition. Ordinate, first order rate constant of the thrombin inactivation. Abscissa, amount of thrombin inactivated in the first phase of the experiment.

serum was kept in a refrigerator at +4°C. A slight decrease of about 5 to 10 per cent was observed in the first 24 hours, but no further diminution of the activity was apparent in the period of time investigated. Also the activity of serum did not change during a period of 3 month's storage in the frozen state at −15°C. This result is in accord with the recent findings of Seegers and associates (26). Stefanini (27), on the contrary, reported an increase in the antithrombic activity of plasma on storage. Probably this apparent activation was due to a shift of the pH of plasma during storage toward the pH optimum of the inactivation.

The heat stability of the factor was investigated at pH 7.23. A mixture of

0.8 ml. serum, 0.2 ml., 0.5 M phosphate buffer, and 0.4 ml. of saline was heated for 5 minutes at different temperatures, then rapidly cooled. The activity of the heated sera was determined by adding 30 units of thrombin and following its disappearance. As can be seen in Fig. 4, the antithrombic factor is fairly stable for the period of 5 minutes below 58°C., but deteriorates rapidly above this temperature. 5 minutes of heating at 65°C. destroys completely the activity (5, 12, 28). At 60°C. the inactivation of the antithrombic factor followed a first order reaction course with $k = 3.5 \times 10^{-3}$ (calculated with natural loga-

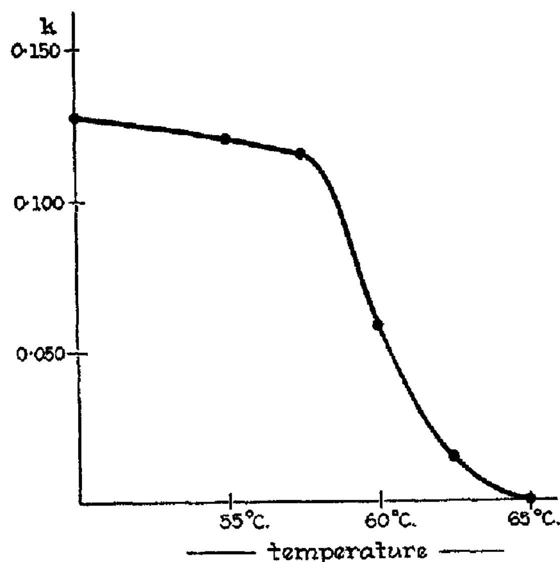

FIG. 4. Heat inactivation of the antithrombic factor. Ordinate, first order rate constant of the thrombin inactivation. Abscissa, temperature of the 3 minute heating.

rithms and time in seconds). From the heat inactivation an approximate energy of activation of 121,000 cal. can be calculated, a value suggesting the denaturation of a large protein molecule.

Effect of Variations in Thrombin and Serum Concentration, pH, and Temperature upon the Rate of Thrombin Inactivation.—The thrombin concentration was varied from approximately 7 to 28 units in 1.5 ml. of reaction mixture. This fourfold increase of the thrombin concentration caused a drop in the reaction rate of approximately 12 per cent.

Varying the serum concentration by diluting it with 0.15 M NaCl solution affects the rate appreciably. The lines shown in Fig. 1 were obtained with serum concentrations of 0, 20, 40, 60, 80, and 100 per cent. There is a slight inactivation of thrombin even in the absence of serum, caused by the absorption

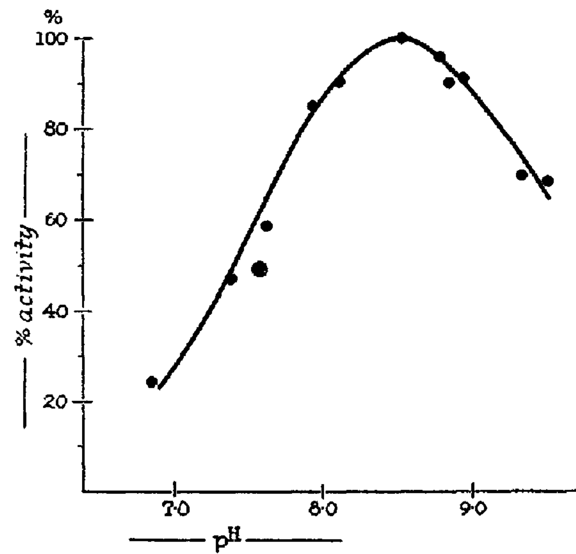

Fig. 5. The rate of the thrombin inactivation as a function of pH. Ordinate, first order rate constant. Abscissa, pH.

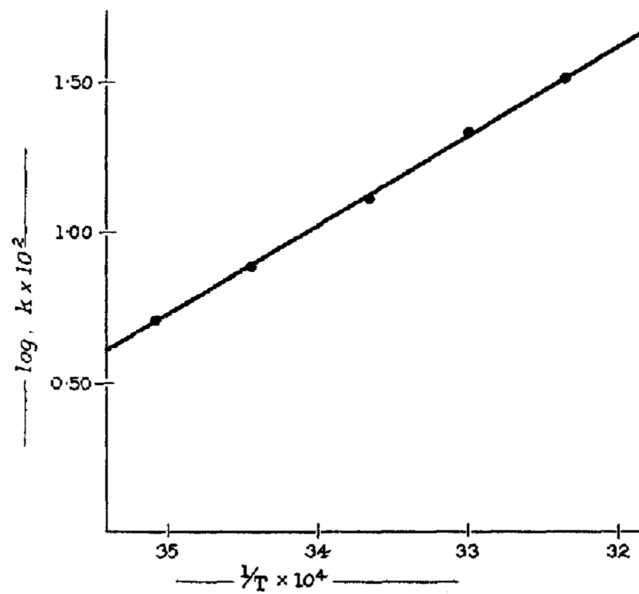

Fig. 6. Arrhenius plot of the rate constant of thrombin inactivation. Ordinate, log of the first order constant. Abscissa, reciprocal of the absolute temperature.

of thrombin on the glass surface, as pointed out recently by several authors (29, 30, 26). The rate constant was proportional to the serum concentration (see also Quick (12)). Under the experimental conditions described, undiluted, normal human sera gave values of k ranging from 0.086 to 0.135, the average of 6 individual sera being 0.116.

Serum samples were adjusted to different pH values with 0.1 N NaOH and 0.1 N HCl, the pH of the mixtures being checked with the glass electrode. No pH change occurred during the inactivation of thrombin. This procedure was used instead of adding buffers in order to avoid excessive pH differences in the second stage of the determination. pH control in the second stage showed that the pH of the fibrinogen solution after addition of the thrombin-serum mixture varied between 6.81 and 7.18, being thus in the range where clotting time is practically independent of pH. Fig. 5 shows the pH dependence of the rate constant of the thrombin inactivation. A clear maximum is apparent at pH 8.5.

A rise in temperature increases markedly the rate of the reaction. The Q_{10} is 1.87 for the temperature range 10–20°C. and 2.42 for the 20–30°C. interval. Fig. 6 shows the Arrhenius plot of the data, log k plotted against $1/T$. From the slope of the straight line an activation energy of 14,000 cal. can be calculated. This value is in agreement with that found earlier by Glazko and Ferguson (20) for the spontaneous inactivation of impure thrombin preparations. The low activation energy certainly indicates that the process is not a protein denaturation.

Effect of Some Added Reagents upon the Rate of Thrombin Inactivation.—

Heparin.—Since its discovery, heparin has been associated with the antithrombic potency of plasma, but in spite of numerous investigations in this field, the problem is still far from being completely clarified. With small thrombin concentrations the rate of inactivation by serum is tremendously increased by heparin in a concentration of a few micrograms per milliliter (21). Fig. 7 shows the increase of the rate as a function of the heparin concentration, with 30 units of thrombin as substrate. The first order character of the reaction is unaffected by heparin. Protamine sulfate and toluidine blue reverse the heparin effect completely. With protamine, as can be seen in Fig. 8, the effect of heparin is completely neutralized when the heparin-protamine weight ratio is 1:1. Jaques, Charles, and Best (31) found a heparin-protamine ratio of 1:3 at the point of equivalence both in *in vitro* and in *in vivo* experiments, and Cowley and Lam (32) reported a ratio of 1:1 in *in vivo* experiments. In the experiments of Portmann and Holden (33), a much higher ratio of 1:18 was observed. As pointed out by Best and Jaques (34), individual protamine preparations may show widely differing reactivity. Increasing the protamine concentration to fifty times the amount necessary to counteract the effect of 10 micrograms of heparin did not change further the rate of thrombin disappearance. A slight

decrease in the rate of inactivation was apparent only with concentrations of protamine of the order of 1 mg. per ml. However, this effect cannot be attributed to a heparin-protamine antagonism. Protamine in high concentrations has a fibrinoplastic effect, accelerating the clotting of fibrinogen, and through the increase of the reactivity of fibrinogen, it may reduce seemingly the rate of the disappearance of thrombin. Therefore, shortening of the clotting time in

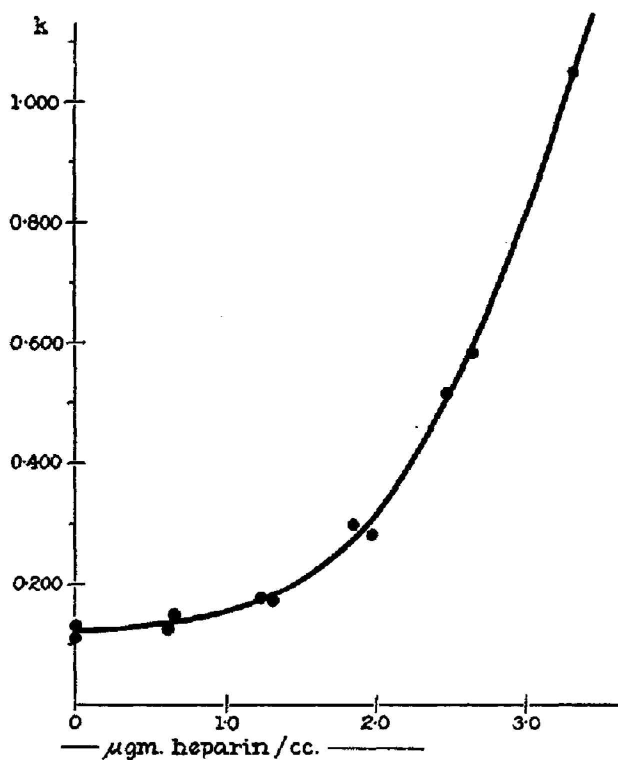

FIG. 7. Effect of heparin on the rate of thrombin destruction by serum. Ordinate, rate constant. Abscissa, heparin concentration.

the presence of fairly high concentrations of protamine does not justify the conclusion of Tanturi and Wetzel (35) on the presence of heparin in normal blood.

The rate increase of the disappearance of thrombin caused by heparin can be suppressed at any time by adding protamine. As can be seen in Fig. 9, the thrombin inactivation after the addition of protamine proceeds at the same rate as in a blank experiment which contains neither heparin, nor protamine.

Toluidine blue in an excess of 7.5 to 10 times over heparin, reduces the rate

of the disappearance of thrombin to the level of the serum without heparin (36). However, contrary to the experience with protamine, increasing the toluidine blue concentration over this value decreases the rate further, below the value of the control. This phenomenon is caused probably by the clotting-accelerating effect of toluidine blue in higher concentrations, described recently by Haley and Rhodes (37). In these experiments the rather acidic toluidine blue was

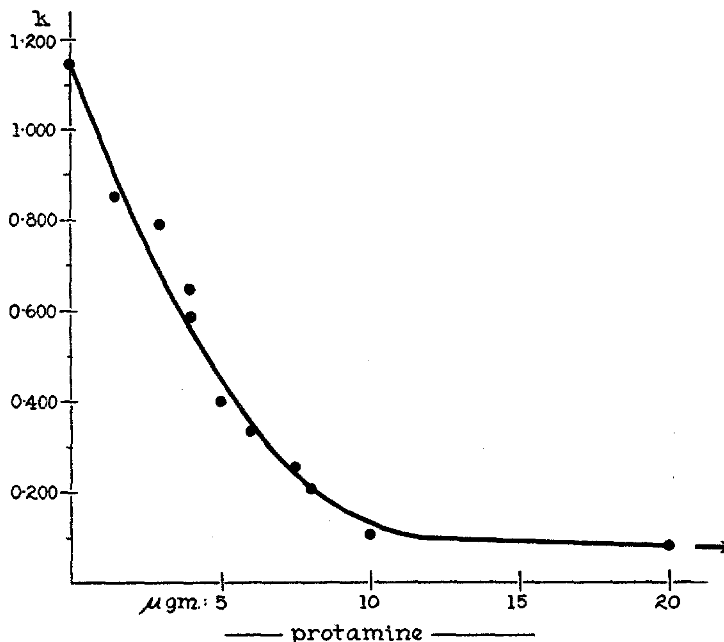

FIG. 8. Heparin-protamine antagonism. Rate constant in the presence of 10 micrograms of heparin and varying amounts of protamine sulfate in the reaction mixture. Ordinate, rate constant. Abscissa, amount of protamine.

carefully neutralized. In general, in all the experiments reported, the pH was controlled rigorously with the glass electrode.

A quite unexpected result was encountered when the effect of heparin was tested on a system with high thrombin concentration. With 300 to 600 units of thrombin as substrate heparin proved to be ineffective in concentrations of 0.006, 0.06, and 0.6 mg. per ml. of reaction mixture. As is shown in Figure 2, the slope of the clotting time *vs.* incubation time plot is increased somewhat by heparin, but this effect is caused by the slight prolongation of the clotting time by heparin in the second stage of the determination, and not by an acceleration of the thrombin destruction. When protamine was added to the samples before the clotting time determination, the two curves had identical slopes.

Histamine.—Gerendás, Csefkó, and Udvardy (38) claimed that histamine in moderate concentration strongly inhibits the thrombin inactivation by serum. In our hands, however, a fairly high histamine hydrochloride concentration of 2.66 mg. per ml. did not affect the rate of thrombin disappearance with normal

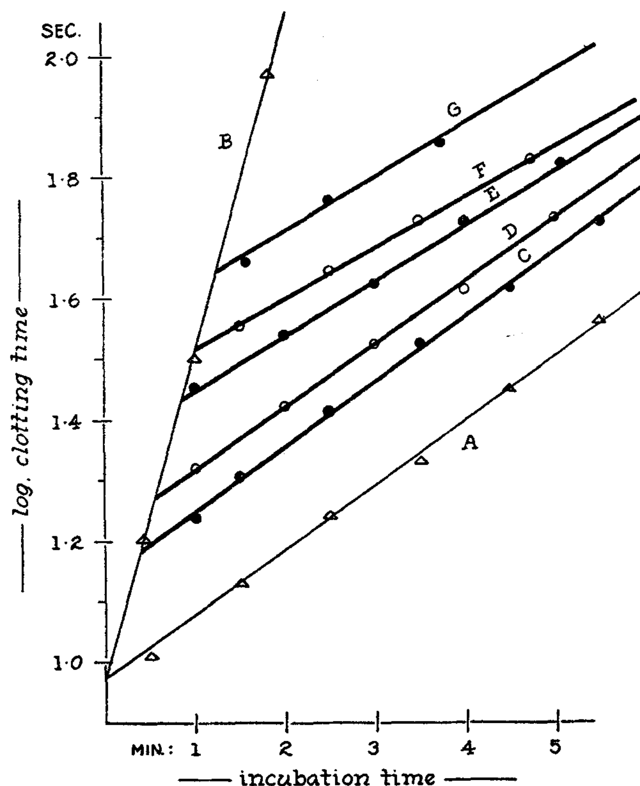

FIG. 9. The increased rate caused by heparin can be normalized by protamine at any time, and from then on the inactivation continues with the normal rate. Curve A, inactivation without heparin. Curve B, with 6 micrograms of heparin. Curves C to G, inactivation in the presence of the same amount of heparin as in curve B, with protamine addition after 20, 30, 50, 60, and 75 seconds respectively. As can be seen, the intersection of curves C to G with curve B corresponds to the moment of the addition of protamine. Ordinate, log of clotting time. Abscissa, incubation time.

serum, neither did it reduce the heparin-induced increase in rate. Monkhous *et al.* (39) came to the same conclusion from *in vivo* experiments. The assumption of a heparin-histamine antagonism with respect to the thrombin inactivation by serum, therefore, cannot be held.

Calcium.—Calcium chloride in 0.02 M concentration increased the rate of

thrombin inactivation by serum by 30 to 50 per cent. However, calcium is not indispensable for the antithrombic activity. Serum incubated with 0.01 M potassium oxalate, or sodium citrate, for a half an hour showed the same activity as an untreated blank.

Heavy Metals.—0.001 M copper sulfate did not change the rate of thrombin disappearance, whereas 0.001 M ferric nitrate slightly inhibited it. These experiments were performed in borate buffer of pH 8.5. Samples of serum were also incubated with several metal-binding reagents: 0.01 M potassium cyanide, or 0.1 per cent sodium diethyldithiocarbamate, did not affect the antithrombic activity. With $\alpha\alpha'$-dipyridyl in a final concentration of 0.1 per cent, a decrease in the thrombin disappearance rate of 27 per cent was observed, but in view of the findings with the previous two reagents, this cannot be considered as due to the removal of heavy metals.

Adsorbing Agents.—Serum treated with several adsorbing agents: barium sulfate, magnesium hydroxide, kaolin, activated carbon, retained its thrombin-inactivating capacity unchanged. Similar findings were also reported by Seegers and associates (26).

α-Tocopheryl Phosphate.—Zierler, Grob, and Lilienthal (40) and Kay and Hutton (41) described an antithrombic effect of α-tocopheryl phosphate. According to the latter authors, a complex is formed from fibrinogen, tocopheryl phosphate, and calcium, which is able to bind large amounts of thrombin. We found also that the disodium salt of α-tocopherol phosphoric acid ester[3] in concentrations higher than 2×10^{-3} M strongly inhibits the clotting of fibrinogen with thrombin, but that the progressive inactivation of thrombin by serum was decreased rather than increased by this reagent. With 2×10^{-3} M concentration the decrease amounted to 38 per cent; with 4×10^{-3} M to 91 per cent of the value of the non-treated serum. However, 0.02 M calcium chloride and 2×10^{-3} M tocopheryl phosphate together slightly increased the rate of inactivation of thrombin in borate buffer of pH 8.5.

Ethyl Alcohol, Ethyl Ether, and Chloroform.—The antithrombic activity of serum is completely inhibited by 20 volume per cent alcohol (42). This inhibition is reversible. Provided the contact with alcohol was not too long, or the temperature too high, the whole activity returned when the alcohol was quickly dialyzed out.

Shaking the serum with ether or chloroform, as previously mentioned by some authors (43, 44), completely and irreversibly destroys the activity.

Soy Bean Trypsin Inhibitor.—The rate of thrombin disappearance was practically unaffected by soy bean trypsin inhibitor. Since plasmin (serum tryptase) is strongly inhibited by the soy inhibitor (45), one may conclude that the destruction of thrombin in serum is not caused by plasmin. This conclusion

[3] Kindly supplied by Mr. Parke Richards, Jr., of Hoffmann-La Roche, Inc., Nutley.

is supported also by the investigations of Glazko (46) and Loomis *et al.* (47), who showed that thrombin is stable in the presence of plasmin.

Dialysis.—Prolonged dialysis against changes of physiological saline solution does not affect the antithrombic factor of serum.

DISCUSSION

The expressions, antithrombin and antithrombic potency, have been used rather indiscriminately in the literature, to designate any substance having a retarding effect upon the clotting of fibrinogen with thrombin. The term antithrombin was reserved in the present paper to designate a specific factor, or possibly factors, present in plasma and in serum capable of inactivating thrombin. The effect of antithrombin is characterized by a progressive destruction of thrombin, the process being irreversible. Earlier authors (1, 2) claimed that thrombin inactivated by serum, the so called metathrombin, can be reactivated by different procedures. However, we are unable to reactivate any thrombin using the different methods proposed for this purpose (1, 2, 48).

As was clearly shown by Seegers and his associates (26, 49, 50), the inactivation of thrombin by serum or plasma leads to an apparent equilibrium (irreversible), dependent on both serum and thrombin concentration. With small thrombin concentrations practically all the thrombin will be destroyed, and in this case, the reaction assumes an apparent first order course. With high thrombin concentrations the reaction proceeds slowly toward an equilibrium with appreciable quantities of thrombin remaining in the active state.

The role of heparin in this system deserves some consideration. Heparin in the presence of a cofactor inhibits the action of thrombin upon fibrinogen. As pointed out by Klein and Seegers (8), this is a dynamic effect and it is not caused by a destruction of thrombin. It is quite distinct from the progressive and irreversible inactivation of thrombin which takes place in serum. As demonstrated in the present paper, the latter process is also affected by heparin if the thrombin concentration in the system is relatively low, but heparin is ineffective in the presence of high thrombin concentrations. Whether the thrombin preparation contained an inhibitor of the heparin which became effective with high thrombin concentrations or whether some other mechanism was involved, cannot be stated at the present time. It is significant that Seegers and associates (26), using their most purified thrombin preparations in relatively high concentration, also found the destruction of thrombin by serum unaffected by heparin.

The effect of heparin in the experiments with low thrombin concentration cannot be explained by an eventual cooperation of the normal antithrombin and heparin plus coinhibitor, the latter having an apparent accelerating effect on the thrombin destruction by prolonging the clotting time in the determination of the residual thrombic activity. By adding protamine to the fibrinogen solution, the effect of heparin mixed with the thrombin solution can be completely

abolished. Nevertheless, the rate of the thrombin disappearance in the presence of heparin was the same whether the fibrinogen solution used for the clotting time determinations contained protamine or not. One may suppose that the neutralization of heparin by protamine requires time or that the serum proteins interfere with the neutralization. To exclude these possibilities, protamine was added after serum, heparin, and thrombin had been incubated for different time intervals, and the rate of disappearance followed from then on. As can be seen in Fig. 9, the neutralization of heparin takes place instantaneously, with the return of the rate to that observed without heparin, but the curves are displaced proportionally with the amount of thrombin destroyed in the presence of heparin. Had the heparin only a dynamic effect, all the curves should coincide with the curve obtained in the absence of heparin.

The rate of thrombin disappearance was always increased by heparin, though with individual sera the magnitude of the effect may have varied by as much as 30 per cent. In the *in vivo* experiments of Monkhous *et al.* (39), the parallelism between k and heparin concentration was not evident, sometimes a high heparin concentration being associated with a normal or even low k value. This was observed with heparin released in anaphylactic shock and also with heparin injected intravenously. Possibly the heparin is released partly in a bound form (51) and the injected heparin may also be bound to some plasma component (52), which may explain the above discrepancy.

From the investigations on the effect of thrombin concentration, pH, and temperature upon the rate of thrombin disappearance, it is evident that these variables should be kept rigorously constant, whenever the rate determination is conducted for the purpose of estimating the concentration of the antithrombic factor. In the latter case the presence of heparin should also be excluded.

SUMMARY

The inactivation of thrombin by serum follows a first order kinetics with thrombin concentrations of the order of 20 to 40 units per ml. of serum. With higher thrombin concentrations of 300 to 600 units per ml. of serum, the rate slows down and not the logarithm of the clotting time, but the clotting time itself increases proportionally with the incubation time.

The antithrombic factor is stable at +4°C. for a period of 2 weeks, or for 3 months at −15°C. Heating over 58°C. destroys it rapidly. The heat inactivation has an energy of activation of 121,000 cal.

With low thrombin concentrations the rate of thrombin inactivation increases linearly with increasing serum concentration. The pH optimum of the inactivation is at 8.5. Increasing the temperature increases the rate. The energy of activation of the thrombin destruction by serum is 14,000 cal.

Heparin increases the rate considerably with low thrombin concentrations, but does not affect the rate with high thrombin concentrations. The effect of

heparin can be abolished with protamine or toluidine blue. Some other reagents were also tested with respect to their effect upon the rate of inactivation of thrombin by serum.

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