Antithrombin I Activity

Its Interference with the Estimation of Antithrombin II Activity in Certain Thrombin Inhibitor Determinations

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Burstein (1, 2) and Nilsson and Wenckert (3, 4) have demonstrated that the in vitro antithrombin activity of heparin is not as high in distilled water as it is in an isotonic salt solution. There is an increase in antithrombin activity with the progressive increase in concentration of the NaCl solvent, i. e. the ionic strength.

Experiments by Graham and Barrow (5) suggest that shortening of recalcification time in progressive plasma dilutions is due to a decrease in ionic strength of the clotting mixture if the ionic strength of the diluent is lower than that of the plasma.

Since a decrease in ionic strength promotes coagulation in vitro and reduces the activity of an exogenous anticoagulant, it is desirable that the ionic strength of the clotting mixture be kept at physiological levels in the investigation of endogenous anticoagulants, lest their activity be underestimated or overlooked.

The experiments to be described were undertaken to determine whether the usual thrombin inhibitor determination meets this demand. The method was described by Jürgens (6): 0.2 ml of a standard thrombin solution is added to 0.1 ml of the plasma to be tested; the fibrin network is removed after 45 seconds; 0.2 ml fibrinogen solution is added to the defibrinated plasma after 60 seconds, and the residual thrombin is assessed on the basis of the clotting time.

Reagents

Plasma: Blood is collected from normal subjects directly in a 3.13% trisodium citrate ($Na_3C_6H_5O_7 \cdot 2H_2O$) solution from a V_2A needle. (9/1 : v/v). The specimen is centrifuged for 15 minutes at 3000 rpm. The plasma is kept at + 2° C until immediately before the test.

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Thrombin: The preparation Topostasin ROCHE was used. The activity of the solution was standardized as originally described by Jürgens (6): 0.1 ml of the thrombin solution clots 0.2 ml of normal human plasma in 12 seconds at 37° C.

Fibrinogen: 60 mg fibrinogen was dissolved in 5 ml NaCl 0.85% and this solution was diluted with 5 ml veronal buffer. During the experiments the solution was stored at 37° C. The majority of the tests were carried out with bovine fibrinogen Poviet*). Instances of the use of bovine fibrinogen Behring are specially mentioned.

Buffers: 1) Veronal buffer Behring pH 7,6 prepared according to the formula given by Schultze and Schwick (7).

2) Veronal buffer pH 7.35, $\mu = 0.154$ (8).

Fibrinolysin: An euglobulin preparation obtained from human serum was activated by a method reported by von Kaulla (9). This fibrinolysin was stored at —20° C, and used within 24 hours. It contains no demonstrable prothrombin, thrombin, fibrinogen, no antithrombin activity and no accelerators of the fibrinogen-fibrin conversion.

Results

The ionic strength of the various reagents used in this thrombin inhibitor determination was calculated from the molarity of the constituents parts according to the equation $\mu = 1/2 \Sigma mz^2$.

To simplify calculations, the following assumptions were made:

1) Dissociation of ions in the reagents was presumed to be complete.

2) The ion concentration of a fibrinogen solution Poviet (600 mg/100 ml) was presumed to equal that of the solvent. This assumption is partly based on the data mentioned in the firm's folder, that a 2% solution in isotonic NaCl solution corresponds with ion concentration 0.15 and partly on the fact that we obtained identical values for solvent and fibrinogen solution at conductivity determinations (t : 25° C).

3) A difference in ionic strength between standardized thrombin solution and the solvent was likewise ignored on the basis of identical conductivity values and, on the other hand, in view of the low ionic concentration found in the thrombin stock solution at specific determinations of the various ions, viz.:

Na: 3 mEq./l.	Ca: no demonstrable quantity
K: no trace	HCO_3: no demonstrable quantity
Cl: 3 mEq./l.	HPO_4: no demonstrable quantity

Only 0.1—0.2 ml of such a thrombin solution was used to prepare 10 ml standardized thrombin solution.

4) Calculation of the ionic strength of citrated human plasma was based on following ionic composition of fresh plasma (10).

*) Poviet productan N. V., Amsterdam.

Na:	142 mEq./l.	Cl:	105 mEq./l.
K:	5 mEq./l.	HCO_3:	25 mEq./l.
Ca:	5 mEq./l.	HPO_4:	2 mEq./l.
Mg:	2 mEq./l.	SO_4:	1 mEq./l.
		proteins:	17 mEq./l.
		organic acids:	4 mEq./l.

Calculations were based also on the following specifications:

1) A haematocrit value of 40—45% after centrifugation at 3000/rpm for 15 minutes. Only blood samples with this value were used.

2) Simplifications such as those introduced by Graham and Barrow (5) with regard to the effect of blood citration, viz.: to ensure (a) that all plasma electrolytes except calcium and citrate were completely ionized, (b) that all calcium — free or bound — reacted with the citrate, while the magnesium did not, (c) that secondary ionization of the phosphate ion could be neglected, and (d) that the only complex formed by calcium citrate was the univalent anion calcium citrate.

The values thus calculated are presented in Table 1.

Table 1: *Calculated ionic strength of different reagents.*

(Formule: $\mu = 1/2\ \Sigma\ m \cdot z^2$. m represents the molarity of each electrolyte and z its valence)

Reagents	Ionic Strength
Veronal buffer Behring pH 7.6	0.057
Veronal buffer pH 7.35	0.154
Citrated human plasma	0.190
Fibrinogen solutions: 60 mg fibrinogen Poviet, dissolved in:	
a) 5 ml NaCl 0.85% and 5 ml veronal buffer pH 7.35	0.1495
b) 5 ml NaCl 0.85% and 5 ml veronal buffer Behring pH 7.6	0.1055

With the aid of these values, the ionic strength for different thrombin inhibitor determinations was calculated (Table 2). For each plasma concentration, two values are given, viz.: the ionic strength at which defibrination takes place and the ionic strength of the final clotting mixture after addition of fibrinogen.

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Table 2: *Calculated ionic strength at various plasma concentrations* as used in the thrombin inhibitor determinations. The upper figures refer to the ionic strength of the defibrinated mixture and the lower figures on the second row refer to the ionic strength of the final clotting mixture.

Technique used	Plasma concentration				
	100%	75%	50%	25%	10%
Original method (J. Jürgens)	0.063	0.052	0.041	0.031	0.023
	0.0605	0.0534	0.0470	0.0412	0.0362
Modification I	0.0636	0.0605	0.0575	0.0544	0.0525
	0.098	0.0961	0.0943	0.0942	0.0913
Modification II	0.1660	0.1632	0.1601	0.1574	0.1552
	0.1596	0.1577	0.1559	0.1540	0.1529

The low ionic strength (particularly that of the defibrination mixture) at which the test is carried out, and the spread of the μ-value over the series of plasma dilutions, are striking. The test arrangement was therefore changed to permit determinations at a physiological ionic concentration, as follows:

Modification I: Veronal buffer Behring pH 7.6 ($\mu = 0.057$) was substituted in all tests by veronal buffer pH 7.35 ($\mu = 0.154$). This arrangement precluded variation of μ in the series of plasma dilutions.

Modification II: Apart from the above modification, thrombin was also dissolved in veronal pH 7.35. Thus defibrination and thrombin determination can take place at a virtually constant, physiological ion concentration.

In Fig. 1 the results of thrombin inhibitor determinations in a series of plasma dilutions subjected to these modifications are compared with the results obtained by the original technique. The curves show that slight modifications in technique produce considerable changes in the results obtained.

Modification I constitutes only a gradual transition between the original technique and modification II, yet the thrombin inhibition in modification I has increased as compared with that in the original technique; in modification II it has been suppressed.

Following investigations were made in order to find an explanation of this discrepancy.

* * *

The above mentioned difference between the two modifications of the method was confirmed without exception in ten different plasma samples. Fig. 2 presents the average values of such determinations. In the case of the highest plasma concentrations (with the highest thrombin inhibitor level), the difference was most marked, showing a progressive disappearance at a plasma concentration of 10—30%. The reverse was seen at lower plasma concentrations, i. e. prolongation of clotting times in modification II.

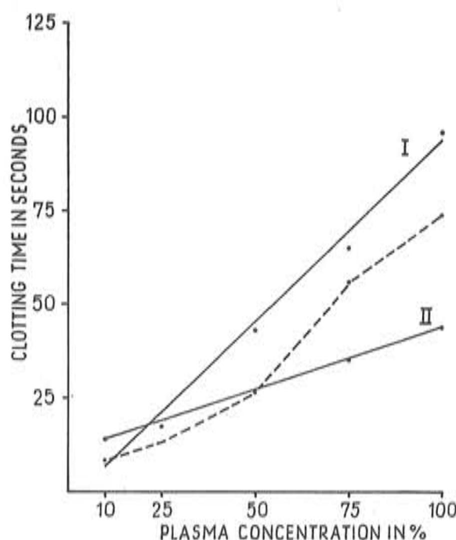

Fig. 1

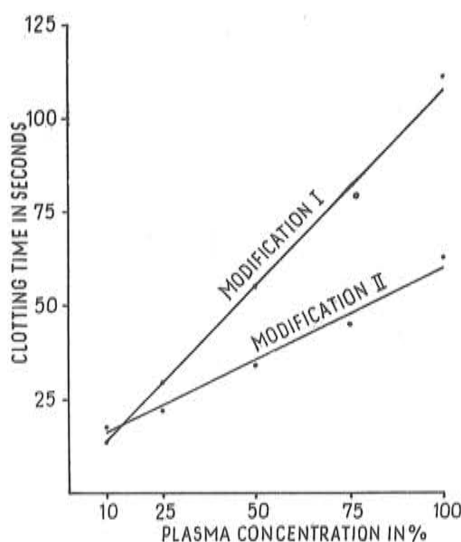

Fig. 2

Fig. 1: *Thrombin inhibitor determinations* in a series of plasma dilutions. Dotted lines: original technique of Jürgens, straight line: modification I: straight line II: modification II (see text).

Fig. 2: *Avarages of thrombin inhibitor determinations* performed in 10 different plasma samples using two modifications of the original technique.

There are various possible explanations for these differences. The first possibility is that clotting times observed in modification II are shorter because a larger quantity of residual thrombin is left. This may be due to inhibition of thrombin inactivation or to the fact that more thrombin was originally added when the experiment was started. It may well be that to attain a standard activity of 12 seconds more units of thrombin were required in veronal buffer than in distilled water. Another possibility is that the residual thrombin in both modifications is constant, but that the difference lies in the rate of reaction of the fibrinogen-fibrin conversion.

These various hypotheses were successively investigated. Table 3 indicates that comparable quantities of thrombin are required to obtain a standardized reagent either in distilled water or in veronal buffer. This fact was repeatedly

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confirmed. In some of the tests the veronal solution was found to contain a somewhat larger quantity of thrombin, but not large enough to explain the difference between the two modifications.

Table 3: *Comparison of the units thrombin required to standardize the solutions* both in distilled water and veronal buffer at pH 7.35:
a) 0.2 ml veronal pH 7.35 + 0.2 ml aqueous thrombin solution are preincubated at 37° C for one minute. Subsequently 0.2 ml fibrinogen solution is added and the clotting time observed.
b) 0.2 ml distilled water + 0.2 ml buffered thrombin solution are preincubated at 37° C for one minute. Subsequently 0.2 ml fibrinogen solution is added and the clotting time observed.

Thrombin concentration in percentage referring to the standardized solution as 100%	Coagulation times of the clottable substrate after addition of: thrombin a)	thrombin b)
100	8"5	6"8
80	10"5	8"2
60	11"5	10"2
40	14"3	12"5
30	16"6	16"5
20	24"3	23"
10	41"4	43"4
5	60"4	63"8
2,5	93"	92"8

Table 4: *Influence of the ionic strength on the fibrinogen-fibrin conversion:*
0.1 ml veronal buffer at pH 7.35 + 0.2 ml thrombin are incubated during one minute at 37° C. 0.2 ml fibrinogen solution is added, and the clotting time assessed.

Thrombin concentration in percentage referring to the standardized solution as 100%	Coagulation times of the clottable substrate after addition of thrombin dissolved in: distilled water	veronal buffer at pH 7.3
100	6"	11"
80	8"	12"4
60	8"4	18"7
50	9"8	19"2
40	11"3	21"
20	18"2	38"2
10	25"2	61"
5	40"	111"
2,5%	55"	144"

In subsequent experiments (Table 4) an attempt was made to determine to what extent the ionic strength — the only difference between the two modi-

fications — influences the rate of reaction at a constant thrombin concentration. With a higher ionic strength inhibition of the rate of reaction was found, increasingly so with a decrease in thrombin concentration. This phenomenon has been repeatedly described (11, 12, 13). Therefore, this does not explain the difference between the two modifications. On the contrary: the difference between the results obtained by the two techniques is bound to be even more marked in reality than is suggested by the values obtained.

The remaining hypothesis mentioned concerns the inhibition of thrombin inactivation. Jürgens claims that his method of determination is specific of thrombin inhibitor activity (heparin antithrombin or active antithrombin II). However the behavior of thrombin inhibition at higher ionic strength (cfr. graphic no 2) is the opposite of the influence of ionic strength on antithrombin activity of heparin added to plasma in vitro (1, 2, 3, 4). The influence of ionic strength on the antithrombin activity of exogenous heparin is studied in Fig. 3 and confirms the literature findings: there is clearly demonstrable increase in activity as a function of the increasing ionic strength of the clotting mixture. The ionic strength of the heparin solvent as such is of no significance (curves 2 and 3). This discrepancy suggests that either thrombin inhibitor determination according to Jürgens is not quite so specific for antithrombin II or the thrombin inhibitor is not of the heparin type.

A systematic investigation was made to determine whether one of the other known antithrombins might interfere with the test and corresponds with the inhibition of activity at higher ionic strength. In view of the short time interval in which the reaction is completed, interference by slow-acting antithrombin III can be excluded. The absence of prothrombin activation in Jürgens' test, on the other hand, excluded antithrombin IV considering that this antithrombin is formed during prothrombin conversion. The remaining possibilities are presence of antithrombin I (fibrin adsorption) or of the antithrombin V recently described by Loeliger (14). Interference from antithrombin I is possible as this is a short-acting antithrombin, and as fibrin formation is included in the process of the reaction. The recently described antithrombin V cannot be excluded either, as the nature of this thrombin inhibitor is similar to that of antithrombin II.

The following experiments were made to investigate antithrombin I interference.

Veronal buffer pH 7.35 was replaced by solutions containing different fibrinogen concentrations, which were used as plasma diluents. Fig. 4 shows thrombin inhibitor determinations in such series of plasma dilutions. At high ionic strength (modification II) the influence was minimal, but at lower ionic

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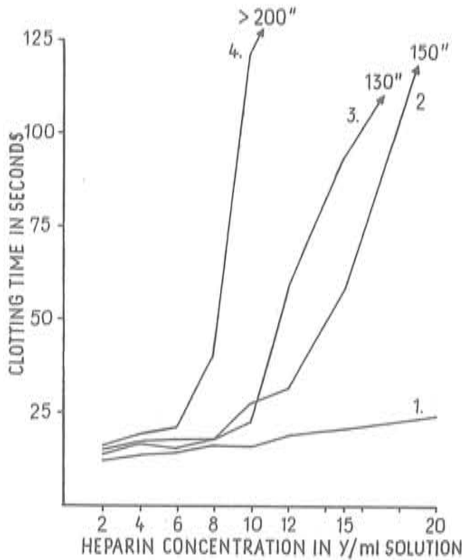
strength (modification I) a marked increase occurred in thrombin inhibition, proportional to the quantity of fibrinogen added.



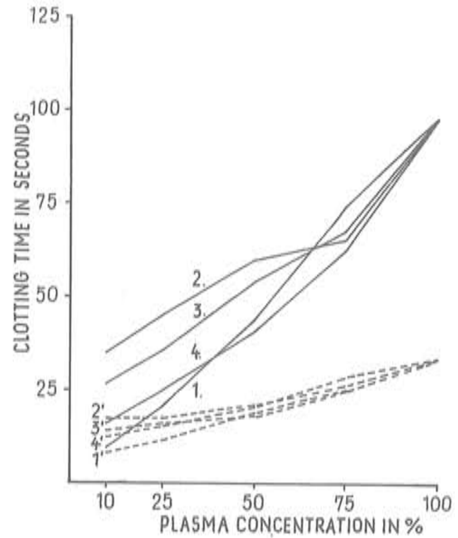


Fig. 4

Fig. 3: *Influence of the ionic strength on the antithrombin activity of heparin* added to plasma in vitro.
(1) heparin and thrombin dissolved in distilled water;
(2) heparin dissolved in distilled water and thrombin in NaCl 0.85%;
(3) heparin dissolved in NaCl 0.85% and thrombin in distilled water;
(4) heparin and thrombin dissolved in NaCl 0.85%.
0.2 ml citrated plasma + 0.1 ml heparin solution + 0.1 ml thrombin solution containing 10 N.I.H. units/ml.

Fig. 4: *Influence of the ionic strength on thrombin inhibitor determinations* in plasma diluted with a fibrinogen Behring solution. Curves 1 to 4 correspond to results obtained by modification I, curves 1' to 4' to results obtained by modifacation II. (1) and (1'): fibrinogen concentration of the diluent 0 mg/ml; (2) and (2') 6 mg/ml; (3) and (3') 4,8 mg/ml; (4) and (4') 2,4 mg/ml.

Figure 5 compares the results obtained by thrombin inhibitor determinations in normal plasma and in plasma from a subject suffering from congenital afibrinogenaemia (15). Thrombin inactivation was considerably less marked in the latter plasma — a result which might suggest more marked thrombin inhibition in normal plasma related to the presence of fibrinogen.

Heating of normal plasma (56° C for 3 minutes) removes fibrinogen and a decrease of thrombin inhibition is also noted. The possibility that the thrombin inhibitor might also precipitate as a consequence of heat defibrination can be ruled out since identical results were obtained in plasma from a case of congenital afibrinogenemia. Other authors (16, 17) have shown that the heparin-cofactorcomplex can withstand these temperatures.

The hypothesis of Witte and Dirnberger (19) according to which fibrinogen is directly related to antithrombin II remains nevertheless important and will be discussed later.

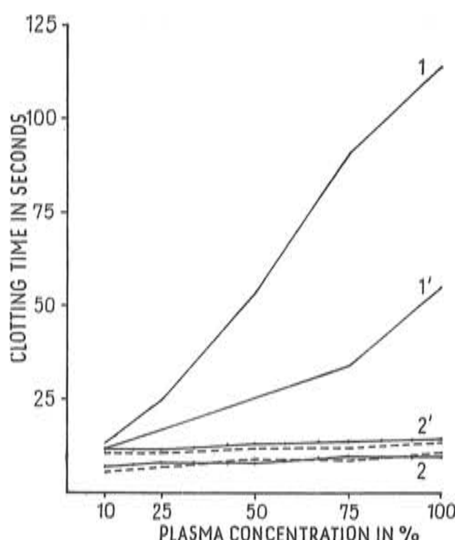


Fig. 5: *Thrombin inhibitor determinations in various plasma concentrations* of a patient with congenital afibrinogenemia (dotted lines) and of normal plasma (straigth lines). Modification I: curves 1, 2 and 3. Modification II: curves 1', 2' and 3'. (1) and (1') normal plasma; (2) and (2') normal plasma defibrinated by heating (3' at 56° C); (3) and (3') plasma of a patient with congenital afibrinogenemia.

Subsequent experiments showed that antithrombin I interferes with thrombin inhibitor determinations according to Jürgens. A fibrinolysin preparation was prepared from human serum in accordance with von Kaulla's technique (9). The fibrin webs. obtained at defibrination in thrombin inhibitor determinations, were added to the fibrinolysin preparation. Thrombin activity in this system was determined following lysis of the clots, on the basis of data provided by Seegers, whereby all the thrombin absorbed onto the fibrin is liberated at lysis (18). The experiments shown in Table 5 demonstrate that the quantity of thrombin liberated after lysis of a fibrin web formed at lower ionic strength is higher than formed at higher ionic strength (modification II). Table 6 gives an estimate of the proportion between the originally added thrombin and the thrombin adsorbed and liberated by lysis. In modification I approximately 50% thrombin is liberated as against only 25% in modification II.

These results explain the difference between the two modifications of Jürgens' thrombin inhibitor determination (Figure 2). At higher ionic

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Table 5: *Estimation of the thrombin activity adsorbed onto the fibrin*

0.4 ml of the standardized thrombin solution is added to 0.2 ml citrated plasma. The fibrin network is whirled on a glass rod, squeezed, removed after 45" and added to 0.5 ml fibrinolysin solution. At the time intervals marked above, the thrombin activity of the sample is measured in the following way: 0.1 ml of the incubation mixture is added to 0.1 ml $BaSO_4$-treated fresh human plasma and the clotting time determinded.

	Thrombin activity liberated from a fibrin web, formed during thrombin inhibitor determination according to Modification I	Modification II
Immediately before lysis of the web	> 600"	> 600"
At time of complete lysis	25"	72"
15 minutes after completion of lysis	22"8	55"

Table 6: *Assay of the antithrombin I activity in comparison with the total thrombin inhibition*

Method: A thrombin solution in veronal buffer at pH 7.35, $\mu = 0.154$ with a fourfold activity compared to the solution recommended by Jürgens is prepared and diluted as mentioned in the table. 0.1 ml of each of these different thrombin dilutions is added to 0.4 ml fibrinolysin solution.
Immediately after mixing and subsequently after an incubation time of 45 minutes at 37° C (time required for complete lysis of the fibrin clot in the former test), the thrombin activity of these samples is assessed by adding 0.1 ml to 0.1 ml $BaSO_4$-treated fresh human plasma.

Thrombin dilutions in percentage. 100% corresponds with a fourfold activity of the usual standardisation	Activity of the fibrinolysin thrombin immediately after mixing	Activity of the same mixtures after incubation during 45 minutes at 37° C
100	12"4	15"5
90	14"2	18"
80	15"5	15"5
70	14"5	19"
60	14"3	21"2
50	19"7	24"2
40	21"6	30"7
30	30"	43"5
20	41"5	61"
10	73"	200"
5	151"	200"

strength the antithrombin I component interfering with the determination is suppressed. The decrease in fibrinogen of progressive plasma dilutions causes

this influence to decrease progressively to a negligible value in the lowest plasma concentrations. At low plasma concentration is the inhibition of the fibrinogen-fibrin conversion at higher ionic strength and constant thrombin concentration manifest and explains why results of modification II exceed those of modification I.

Discussion

Our experiments demonstrate that Jürgens' thrombin inhibitor determination is subject to antithrombin I interference (fibrin adsorption). The same holds true for methods of determination described by other authors (19, 20, 21) using the same principle.

Our results further demonstrate that a decrease in ionic strength of the medium is associated with a considerable increase in the thrombin-adsorbing power of fibrin. It was estimated that the same quantity of plasma fibrinogen adsorbed 50% of a quantity of thrombin at ionic strength 0.07, and only 25% of the same quantity of thrombin at ionic strength 0.154. Further investigations might reveal the connection between ionic strength and antithrombin I activity, the importance of this relationship has been established in this paper.

In a number of normal plasma samples we estimated the portion of added thrombin which is adsorbed onto the fibrinogen during thrombin inhibitor determination according to Jürgens. At ionic strength 0.07 this quantity was 40—65% per 0.1 ml undiluted plasma; at ionic strength 0.154 it was 10—35%. This scatter can probably be explained by the assay character of the antithrombin I estimations and by the difference in fibrinogen content of the plasma samples, which may vary from 250 to 500 mg%. It should be born in mind that, if a constant quantity of thrombin is added to fibrinogen solutions of increasing concentration, a progressive amount will be adsorbed. In view of this relationship and of the antithrombin I interference observed, the spread of thrombin inhibitor determinations in normal plasma samples is understandable.

Preliminary investigations which have yet to be confirmed have shown that the abnormal thrombin inhibitor content found by Jürgens in various pathological conditions can be attributed to fluctuations in the fibrinogen content.

Expressed in percent, the total thrombin inhibition according to Jürgens at ionic strength 0.154 is about 85% and at ionic strength 0.07 more than 98,5% of the thrombin added. Some 25% and 50% of this value respectively, are attributable to antithrombin I activity; the remaining 50% are related to

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immediate plasmatic antithrombin fraction. These values are in. accordance with the thrombin inactivation found in plasma free of the antithrombin I side effect, viz.: normal heat-defibrinated plasma and congenital afibrinogenemia plasma.

The fact that the total direct antithrombin fraction in normal plasma corresponds with the sum of the antithrombin I component (i.e. specifically the quantity of thrombin liberated from fibrin by lysis) and a fraction equivalent to the antithrombin activity in plasma without fibrinogen, makes less plausible Witte and Dirnberger's hypothesis which assumes accepting a close relationship between fibrinogen and thrombin inhibitor.

According to Jürgens, results of thrombin inhibition determined in progressive plasma dilutions give a straight line on semilogarithmic paper. Using our modifications of the method, we were never able to reduplicate these results; there was invariably a linear plot on decimal gradient. Determinations in series of progressive plasma dilutions varying from 100 to 5% at intervals of only 5% confirmed our finding.

The exact cause of this difference is as yet unknown. It may be suggested, however, that it is related to the ionic strength of the dilutions. It should be noted that both Jürgens and Witte and Dirnberger had a linear plot on a semilogarithmic gradient; but both investigators used buffers with a low ionic strenght.

On the other hand, Schmid (21) using Michäelis buffer $\mu = 0.154$, also obtained a linear plot on a decimal gradient.

Summary

1. Our experiments demonstrate that antithrombin I interferes considerably in the thrombin inhibitor determination described by Jürgens. A similar interference can be suspected in the immediate antithrombin determinations as described by other investigators in experiments based on the same principle.

2. The thrombin-adsorbing capacity of fibrin depends to a high degree on the ionic strength of the medium: low ionic strength enhances, while high concentrations inhibite antithrombin I activity.

3. After allowing for antithrombin I interference in the total thrombin inhibition, there was still measurable direct antithrombin activity present. The magnitude of the latter is directly correlated to the antithrombin capacity found in plasma samples free of the antithrombin I side-effect. Such plasma samples were obtained from a patient with congenital afibrinogenemia and heat-defibrinated normal plasma.

Résumé

1. Nos recherches montrent que l'antithrombine I interfère considérablement dans la détermination de l'antithrombine immédiate décrite par Jürgens. Une interférence semblable peut être également suspectée au cours des déterminations de l'antithrombine immédiate décrites par d'autres auteurs.

2. La capacité d'adsorption de la fibrine pour la thrombine dépend beaucoup de la force ionique du milieu: l'activité antithrombinique I est augmentée à faible force ionique, au contraire diminuée aux forces ioniques élevées.

3. Même si l'on tient compte de l'interférence de l'antithrombine I dans l'inhibition totale de la thrombine, il reste toujours une activité antithrombinique directement mesurable. Le taux de cette dernière est en corrélation étroite avec la capacité antithrombinique trouvée dans les plasmas libres des effets secondaires de l'antithrombine I. Les plasmas de patients ayant une afibrinogénémie congénitale et les plasmas normaux défibrinés sous l'action de la chaleur remplissent ces conditions.

Zusammenfassung

1. Unsere Versuche zeigen, daß das Antithrombin I die Bestimmung des Thrombin-Inhibitors nach Jürgens ziemlich stark beeinflußt. Eine ähnliche Interferenz kann vermutet werden für die Bestimmungen des sofort wirksamen Antithrombins, wie sie von anderen Autoren beschrieben wurden, auf Grund von Versuchen, die auf demselben Prinzip beruhen.

2. Die Thrombin-Adsorptions-Kapazität des Fibrins hängt in einem hohen Grade ab von der Ionenstärke des Milieus: Tiefe Ionenstärke steigert, hohe dagegen hemmt die Antithrombin-I-Aktivität.

3. Auch wenn man die Interferenz des Antithrombin I bei der totalen Hemmung des Thrombins in Betracht zieht, bleibt immer noch eine meßbare direkte Antithrombin-Aktivität. Die Größe der letzteren steht in direkter Beziehung zur Antithrombin-Kapazität, die in Plasmen gefunden wird, die frei sind von Nebenwirkungen des Antithrombin I. Solche Plasmen wurden erhalten von Patienten mit kongenitaler Afibrinogenämie und durch Hitzedefibrinierung normaler Plasmen.

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