Thrombin Formation in Purified Systems

Department of Biological Chemistry, Harvard Medical School and Protein Foundation, Boston, Mass., USA)*

Douglas M. Surgenor, Barbara B. Steele and Donald F. H. Wallach

At least five constituents have now been implicated strongly in the initial stages of coagulation. These are the platelets, antihemophilic factor (AHF, VIII), plasma thromboplastin component (PTC, Christmas Factor, IX), Factor V (Ac Globulin) and calcium. Recognizing the kinetic complexities which would be associated with a single reaction involving all five reactants, Biggs, Douglas and Macfarlane (4) made the attractive suggestion that the process may consist of a series of intermediate reactions, the end product of which acts upon prothrombin and is therefore conveniently referred to as blood prothrombin activator or blood thromboplastin.**) These workers investigated possible steps in the sequence and concluded that platelets and PTC shared a common property in being affected by exposure to glass while AHF and Factor V were not. It was further suggested that one preliminary reaction might involve PTC and Factor V since more rapid evolution of thrombin followed preincubation of platelet-poor citrated hemophilia plasma in glass tubes. Bergsagel (1) later postulated an interaction between calcium and PTC which was enhanced by AHF and reversed by dialysis or treatment with chelating agents. This was thought to be an intermediate product in the formation of blood prothrombin activator. More recently Bergsagel and Hougie (3) adopted the designation "Product I", for the hypothetical intermediate formed on incubation of calcium, normal serum and $Al(OH)_3$ treated normal plasma, and went on to search for the simplest intermediate of which the platelet was a part. They applied the criterion of sedimentability and found

*) This work was supported by a grant (H-2440) from the National Institutes of Health, U.S. Public Health Service, from the Kresge Foundation and other charitable trusts and by contributions from companies interested in the alleviation and cure of human diseases.

**) The designation 'blood' is retained to distinguish the product from the similar (and possibly identical) product derived from tissue and blood factors (5).

that the simplest platelet-derived active sediment was obtained when platelets were incubated with congenital Factor VII deficient serum (active in PTC), purified bovine AHF and calcium. They refer to this intermediate as "Product I — treated platelets".

In a highly purified system we have obtained evidence which sheds further light on the mechanism of this complex system, especially on the role of platelets, antihemophilic factor and PTC. It has further been shown that an interaction between calcium ions, platelets and PTC results in alteration of PTC and is one of the earliest events in the clotting sequence.

Experimental

Human Platelets were obtained from fresh ACD blood using a differential centrifugation technique (10, 12); as received, they were sterile and had been washed and suspended for preservation in a dextrose-acetate-gelatin medium. Platelets were usually used without further treatment. The age of the platelets was generally less than three months, except where noted. Except for differences in count, — those preparations with high platelet counts were favored for therapeutic use — these platelets are representative of those in routine intravenous use (11). The composition was, on the average, approximately 400 000 platelets, 100 leucocytes, 1 000 erythrocytes per mm^3 of suspension. Platelet counts were performed in duplicate using a phase microscope and siliconed pipettes.

Plasma thromboplastin component (PTC) was prepared from human serum by the method of White, Aggeler and Glendening (14). The final dried product exhibited corrective activity when tested with the plasma from a patient with PTC deficiency. Thus, substitution of dried PTC for a buffer control in the system: 0.1 ml frozen PTC deficient plasma, containing added platelets, purified AHF and Factor V, 0.1 ml buffer or test solution and 0.1 ml. 0.025 M calcium, reduced the clotting time from 81.1 ± 3.2 sec. to 28.3 sec. at 37°. Freedom from contamination by Factor VII was shown by the complete lack of effect on the one-stage prothrombin time of PTC deficient plasma; control 20.2 sec., test 20.2 sec. Aged human serum (1 : 10) resulted in reduction of the test time from 20.2 to 14.3 sec.

Antihemophilic Factor (AHF) was prepared from fresh platelet-poor resin plasma following treatment with barium sulfate (6). The activity was precipitated into Fraction I. It was then treated with dextran sulfate by a procedure to be described, to yield AHF with only traces of fibrinogen (8). The AHF so prepared had no detectable PTC activity as revealed by tests on the clotting

time of PTC deficient plasma; and was devoid of prothrombin, Factor VII and Factor V activity. It was kept as a dried powder, and proved to be stable indefinitely at — 30°.

Factor V (Ac Globulin) was also isolated from fresh platelet-poor resin plasma following $BaSO_4$ adsorption of prothrombin. A zinc fractionation procedure proved most effective for concentrating this factor; the final fraction comprised less than 1 per cent of the plasma protein. Details of the method will be published separately (9). Factor V so prepared was extremely stable in the dried state, and was free of contamination of all other clotting activities.

Prothrombin was prepared from the same plasma source by one of several procedures. Although prothrombin eluted from barium sulfate has proved satisfactory, we have recently discovered that if the pH is even slightly acid during the elution there is an important, albeit subtle, change in the prothrombin. We therefore prefer to avoid the $BaSO_4$ step and purify prothrombin from Fraction III (7); in the procedure used, the Factor V activity is removed quantitatively, but small amounts of PTC, and presumably also Factor VII, persist in the final product. This was kept as a dried powder, and had an activity of 80—100 units per milligram.

Reaction system. A modified two-stage reaction system was used for most experiments (7). The reaction was conducted at 28° in a bath. In a typical experiment, the composition was as follows: 0.18 ml of Ca-Mg. reagent ($CaCl_2$ 0.035 M; $Mg(OAc)_2$ 0.058 M); 0.2 ml platelet suspension (ca. 400 000/mm^3); 0.1 ml dried AHF (1 mg/ml); 0.1 ml dried Factor V (2 mg/ml); 0.1 ml dried PTC (1 mg/ml) and 2.82 ml of pH 7.4 imidazole (0.05 M) sodium chloride (0.066 M). Finally 0.5 ml of prothrombin solution, 16.8 μg ± 0.8 μg/ml, was added and the stop watch for timing the reaction was started. The total protein concentration in the reaction mixture was approximately 1 mg/ml. At desired intervals, aliquots of 0.4 ml were transferred to clotting tubes containing 0.1 ml of bovine fibrinogen (5 mg/ml) for assay of the thrombin formed. At the low initial prothrombin concentrations here used, the reaction of conversion to thrombin is first order with respect to prothrombin. For this reason, the initial prothrombin concentration was kept the same in all experiments.

Lusteroid tubes and siliconed pipettes were employed in the experiments where platelets were preincubated with the various plasma factors.

Results

The reaction system described above is the simplest one which will yield thrombin under the experimental conditions used. When any one reagent is

eliminated from the reaction mixture, thrombin formation does not occur, or proceeds at a vanishingly small rate. The time course of thrombin formation is shown in Figure 1. An induction period is followed by a rapid generation of thrombin that tapers off and approaches the theoretical yield of thrombin. In contrast to plasma or serum, the system is quite stable, and lacks antithrombin or other inhibitors of thrombin formation.

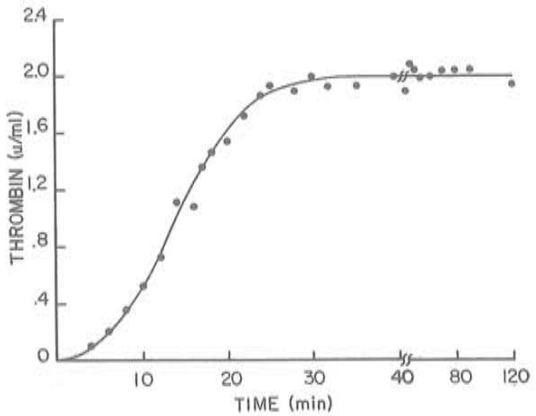

Fig. 1: Thrombin generation from purified human reactants; platelets, factors V, VIII (AHF), IX (PTC), and prothrombin in the presence of calcium. Initial prothrombin concentration 2.0 u/ml.

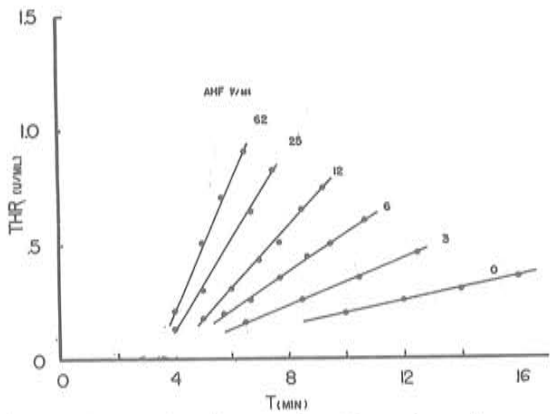

Fig. 2: Effect of AHF (VIII) on thrombin generation. The numbers adjacent to each curve refer to the concentration of added AHF in μg/ml of reaction mixture.

The response of this system to antihemophilic factor (AHF) is shown in Figure 2. The striking feature is that increments of AHF alter the slope of the second phase of the reaction but do not have any significant effect upon the

length of the induction period.*) This implies that one or more reactions precede the formation of prothrombin activator and that the reaction in which AHF participates does not determine the overall rate of activator formation. Further, the initial reactions are probably slow in comparison to the final reaction catalyzed by prothrombin activator. These tentative conclusions are in keeping with the hypothesis of a sequence of reactions leading to blood prothrombin activator formation (4).

The velocity of thrombin formation taken from the slope of the linear portion of the curve, is plotted as a function of the concentration of AHF in Figure 3. Since AHF possesses no prothrombin activator activity per se, it is reasonable to conclude that AHF determines the *amount* of activator formed. This conclusion is supported by the finding of a quantitatively similar response of the same prothrombin to the system: brain tissue extract, Factor VII, Factor V and calcium. The limiting velocity with either blood or tissue activator probably represents the maximum rate of thrombin formation at the prothrombin concentration and under the conditions employed. This relationship between velocity of thrombin formation and AHF concentration forms the basis of a quantitative assay for AHF (8).

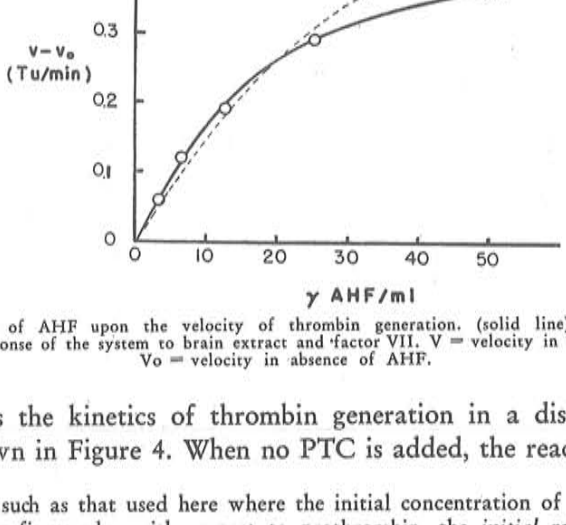

Fig. 3: Effect of AHF upon the velocity of thrombin generation. (solid line). The dotted line represents the response of the system to brain extract and factor VII. V = velocity in thrombin units/min; Vo = velocity in absence of AHF.

PTC affects the kinetics of thrombin generation in a distinctly different manner, as shown in Figure 4. When no PTC is added, the reactions proceed as

*) In a system such as that used here where the initial concentration of substrate is limited, i.e., the reaction is first order with respect to prothrombin, the *initial* rate of formation of product provides the most accurate measure of enzyme activity. We have not ordinarily measured thrombin formation beyond this stage of reaction.

in Curve 1. The basal thrombin generation is attributed to contamination of the prothrombin with PTC*). The AHF concentration used in this experiment corresponds to 62 μg/ml (cf. Figure 2). Addition of PTC results in Curve 2; the induction period was reduced and the linear phase of the thrombin generation curve became normal. This data suggested that PTC is involved in one of the postulated initial reactions. Evidence in confirmation of this was obtained from preincubation experiments. Of all the possible combinations of reactants, the simplest to show a significant effect was the system: platelets; PTC; calcium. Preincubation of these reactants for ten minutes at 37° resulted in the data plotted in Curve 4, in which the induction period is shortened by two minutes. This effect was lost if any one of the three reactants was absent during the preincubation period, cf. Curve 3 where calcium was omitted. The identical slopes of Curves 2, 3 and 4 confirm the suggestion that AHF participates in a distinctly different way from that in which PTC participates.

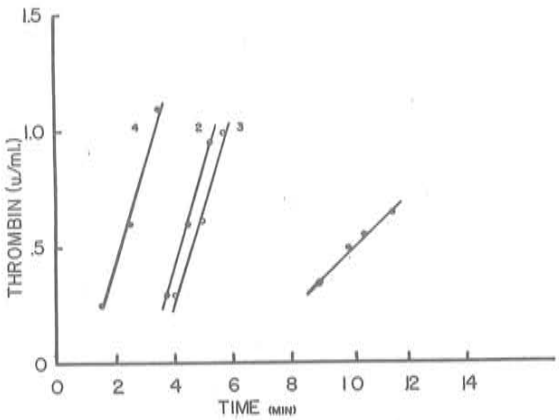

Fig. 4: Thrombin generation in a system limited by PTC (IX) (Curve 1). Curve 2: response to added PTC. Curve 3: PTC and platelets preincubated 10 min. at 37° before addition of other reagents. Curve 4: PTC, platelets and calcium preincubated 10 min. at 37 °.

An attempt was made to isolate an active product of the platelet, PTC, calcium reaction by centrifugation. In order to do so, it was first necessary to establish the behavior of the clot promoting activity of platelets alone. Some data are presented in Figure 5. After sedimentation for thirty minutes at 24 000 x g the supernatant still had appreciable activity when supplied to a complete thrombin generating system. It was necessary to centrifuge at 81 000 x g for forty-five minutes in order to remove the platelet activity

*) We have selected prothrombin preparations for low activity in this system. Different lots of prothrombin vary in their reactivity and therefore presumably in the extent of their contamination by PTC. However, all lots used in these experiments have exhibited the quantitative response to PTC which is shown here.

completely. Accordingly a platelet, PTC, calcium reaction mixture was incubated for ten minutes at 37° in a lusteriod tube and then was centrifuged at 81 000 x g for forty-five minutes. Like the starting PTC reagent, the supernatant lacked prothrombin activator; it was completely inert when antihemophilic factor, Factor V and prothrombin were added. When fresh platelets were also supplied (or indeed if the original platelets were left in the system) prothrombin activator was formed but with the kinetics characteristic of the preincubated platelet, PTC, calcium system (short induction period) Curve 4, Figure 4.

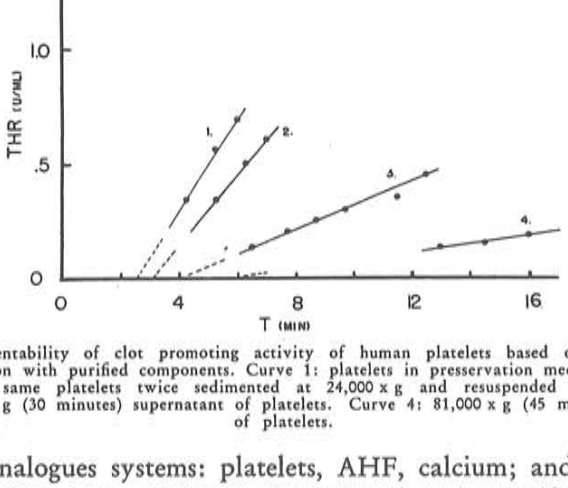

Fig. 5: Sedimentability of clot promoting activity of human platelets based on observations of thrombin generation with purified components. Curve 1: platelets in presservation medium used in usual way. Curve 2: same platelets twice sedimented at 24,000 x g and resuspended in fresh medium. Curve 3: 24,000 x g (30 minutes) supernatant of platelets. Curve 4: 81,000 x g (45 minutes) supernatant of platelets.

When the analogues systems: platelets, AHF, calcium; and platelets, Factor V and calcium, were similary preincubated and centrifuged, no kinetic modification occurred although the 81 000 x g supernatants exhibited the expected AHF and Factor V activities respectively. These experiments suggest that PTC is changed by incubation with platelets and calcium into a kinetically more reactive form or 'activated' PTC. Activated PTC is not formed in the absence of calcium under the conditions of our experiment, nor is it formed by incubating PTC and calcium in the absence of platelets. Studies on the nature of this transformation are now in progress. The activity is stable for days in solution at 0°. Following dialysis and restoration of the calcium, the activity remained unchanged. These observations contrast with previous findings of Bergsagel (1) regarding activation of the Christmas Factor in whole serum by calcium alone (slowly) and by calcium and AHF (rapidly); but not by platelets and calcium.

The effect of activation of PTC in reducing the induction period in the

system of purified components gave rise to the suggestion that a similar effect might be observable in whole plasma under the proper conditions. This proved to be the case. PTC in 0.1 ml of buffer, 0.1 ml of platelets and 0.1 ml of 0.025 calcium chloride were incubated ten minutes at 37° and then added to 0.1 ml of fresh normal plasma. The clotting time was twenty-four seconds. A control without PTC gave a clotting time of seventy-five seconds. This experiment represents indirect confirmation of the activation phenomenon observed in the isolated system. Bergsagel and Biggs (2) have postulated an intermediate (Product I) formed by interaction of AHF, calcium and normal serum which shortens the clotting time of platelet-containing plasma; serum from a patient with Christmas disease lacked the property to participate in this reaction. We cannot confirm this result with our purified clotting factors.

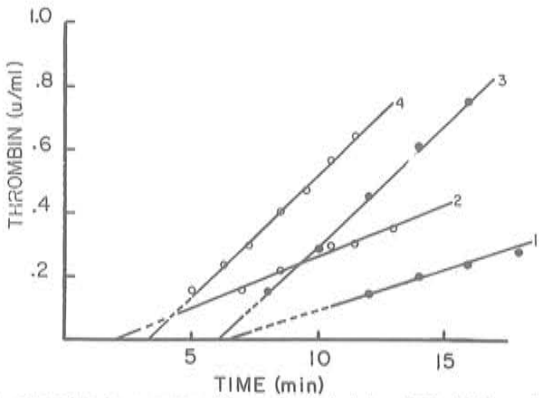

Fig. 6: Thrombin generation in a system (Curve 1) limited by AHF (VIII) and factor V, Curve 2 limited by AHF only. Curve 3 limited by factor V only and Curve 4 unlimited by either.

Factor V appears to participate in a manner analogous to PTC, as shown by the data in Figure 6. Factor V affects the relative displacement of the thrombin generation curve on the time axis rather than the slope of the curve. In these experiments, the system was also limited by PTC. Under these conditions the data suggest that the system could be saturated with respect to Factor V since doubling the amount used had no additional effect upon the response.*)

*) The similarity in response resulting from addition of PTC and Factor V raises the question of whether the Factor V could be contaminated by PTC (the reverse could certainly not be true). This seems highly unlikely in view of the fact that Factor V does not correct the defect in PTC-deficient plasma. Furthermore, Factor V is prepared from barium sulfate adsorbed plasma by a procedure which, even though the adsorption step is omitted, yields a product which contains only traces of prothrombin. However, lacking a preparation of PTC made from *plasma*, and therefore lacking knowledge of the properties of plasma PTC, we cannot make a positive statement.

Proceeding on the now very likely hypothesis that prothrombin activator is formed by a series of reactions, and assuming that a platelet, PTC, calcium reaction represents an early step, it should be possible to illuminate some of the later reactions by manipulation of the relative concentrations of reactants. This in effect has already been done for AHF in Figure 2 and for PTC in Figure 4. When the system was limited by both AHF and PTC, the results shown in Figure 7 were obtained. The concentration of reactants is the same as used for

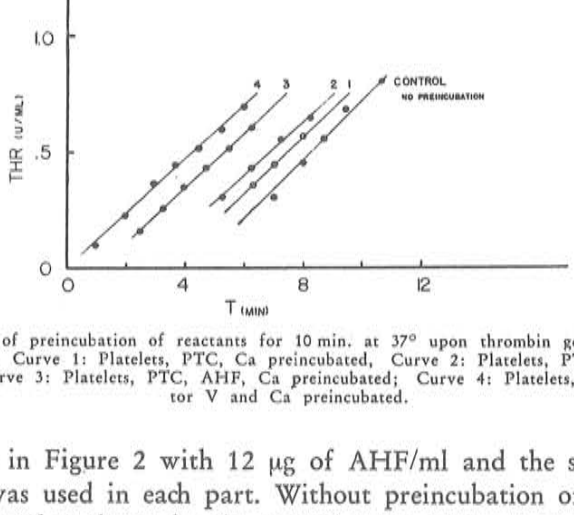

Fig. 7: Effect of preincubation of reactants for 10 min. at 37° upon thrombin generation. Control: no preincubation; Curve 1: Platelets, PTC, Ca preincubated, Curve 2: Platelets, PTC, factor V, Ca, preincubated; Curve 3: Platelets, PTC, AHF, Ca preincubated; Curve 4: Platelets, PTC, AHF, factor V and Ca preincubated.

the experiment in Figure 2 with 12 μg of AHF/ml and the same amount of each reactant was used in each part. Without preincubation of any reactants, thrombin is formed as shown by the control curve. There is an apparent induction period of four minutes. Curve 1 represents the effect of activating the PTC by preincubation (10 minutes at 37°) with platelets, with PTC and calcium, after which the remaining reagents were added at zero time. When Factor V is added to the preincubation mixture, there is a slight further reduction in the induction period. Curve 3 illustrates the result when platelets, PTC, calcium and AHF are preincubated; the induction period is markedly reduced in comparison to the control. Finally when all components except prothrombin are allowed to react (Curve 4) thrombin formation is almost instantaneous when the substrate is added. This implies that the product of the interaction in experiment 4 was a prothrombin activator which can act directly upon prothrombin. Alternatively, it was kinetically very close to the final activator in the reaction sequence. The intermediate product of reaction 3 may correspond to Product I-treated platelets (3).

The kinetic aspects of platelet participation in the formation of thromboplastin have not heretofore been studied. In the intact thrombin generation system, the response to variation in platelet numbers is shown in Figure 8. The

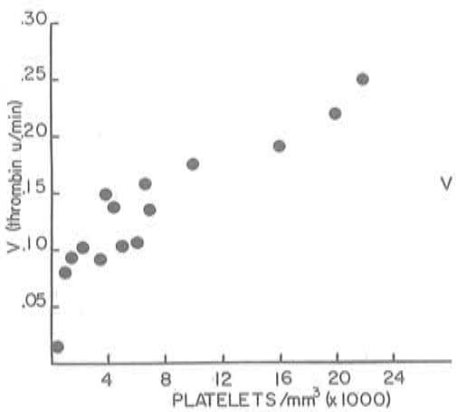

Fig. 8

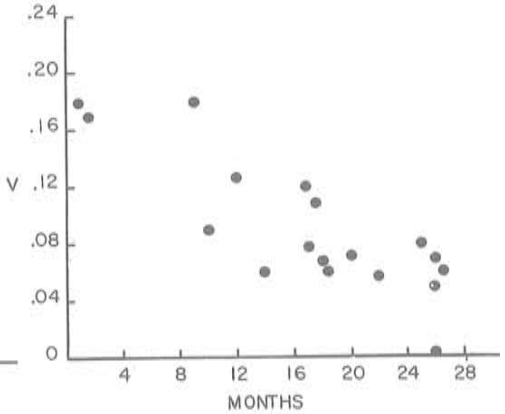

Fig. 9

Fig. 8: Effect of platelet concentration on velocity of thrombin formation. A single unit of human platelets which had been preserved for 4 months was used in these experiments.

Fig. 9: Effect of platelet age on velocity of thrombin formation. The platelet concentration was between 10,000 and 20,000/mm³ in each experiment.

response is very sensitive when the number of platelets is less than 1500 per mm³. Above this level the system appears to be saturated or nearly saturated with respect to platelets. The two ranges of platelet concentration are further differentiated by the effect on the induction period. Up to 1000—1500 platelets/mm³ the addition of platelets increases the velocity of the linear thrombin generating phase and decreases the induction period. Beyond this range the effect is restricted to increase in the velocity of thrombin generation, i.e., the PTC system appears to be saturated. (At 1000 platelets/mm³ and 25 μg of PTC/ml, assuming a molecular weight of 250 000 for PTC and that the preparation contains only one per cent of PTC, there are approximately 100 000 molecules per platelet.) As a part of a study on platelet preservation (11), platelets were tested which had been preserved for varying periods up to thirty months. At the time of the experiments the concentration of platelets in the reaction mixture varied somewhat, but was always in the range of 10 000—20 000/mm³ of reaction mixture, i.e., in the range of saturation. Certain morphologic changes occur during the long preservation period, including changes in the number of cells with spicules and blebs. In contrast, however, the standard units of preserved platelets do not change greatly in platelet *count* during preservation, nor is there significant tendency to aggregate. Despite this, the data presented in Figure 9, reveal a gradual decline in activity, as indicated by the

velocity of the thrombin generation which they engender. In addition to this effect, the data also reveal that the induction period is increased as the platelets age. These findings suggest that there are qualitative changes in platelets during preservation which manifest themselves quite apart from quantitative changes in numbers or frequency distribution of various forms within the population.

Discussion

The results obtained thus far are in good agreement with the postulated series of interactions in the formation of prothrombin activator. The observed change (activation) in PTC following treatment with platelets and calcium is characterized by a striking reduction in the time necessary for prothrombin activator to form and thrombin generation to proceed at a linear rate. PTC so treated *still requires the presence of platelets;* if the platelets are removed following the activation stage, prothrombin activator cannot be formed. This implies that platelets are involved at two levels of the sequence: in the activation of PTC and in a later step. Our data do not exclude a reaction equivalent to Product I-treated platelets, although they do not appear to support the concept of Product I. In this connection it appears that the postulated existence of Product I depended upon observations made with aluminium hydroxide absorbed plasma and normal serum as sources of AHG and PTC respectively (4). The later experiments on Product I-treated platelets made use of purified factors but do not appear to include confirmation of Product I (3). On the other hand, we are cognizant that the activation of PTC which we have observed may be relatively insignificant in terms of the physiological formation of prothrombin activator, in that we have used a partially purified serum PTC preparation which has been exposed to strong metal chelating conditions and acid pH. Despite suggestions to the contrary (1), it does not seem to have yet been excluded that PTC exists in the circulation as an 'active' calcium complex. Studies now in progress in our laboratory on the purification and characterization of PTC from both plasma and serum should illuminate this point. The activation of PTC observed here is produced equally well by lipid extract of platelets (13). Studies in this direction will be reported separately.

On the basis of these experiments with highly purified reagents we view the primary event as the activation of PTC. Because of the long induction period when PTC is not previously activated, it appears that the activation process is slow relative to later steps. The data further suggest the existence of at least one other reaction (and therefore another intermediate product) prior to the formation of the final activator. In contrast to the PTC-involved step,

AHF appears to determine the *amount* of activator. In either case, the final reaction between prothrombin activator and the substrate prothrombin is probably very fast in comparison to the preceding ones.

Summary

Study of the kinetic behavior of purified human clotting factors suggests that formation of prothrombin activator from platelets, plasma thromboplastin component (PTC), antihemophilic factor (AHF), Factor V and calcium proceeds by a sequence of reactions. The earliest detectable reaction is slow and involves platelets, PTC and calcium; an intermediate activated PTC is obtained which is free of platelet contamination and is characterized by its kinetic behavior. Platelets are also requisite at a later step in the sequence, possibly in an intermediate formed between activated PTC, AHF, platelets and calcium. Variations in platelets, AHF and PTC result in characteristic responses in the kinetics of thrombin formation.

Résumé

L'étude du comportement de certains facteurs de coagulation fait admettre que la formation de l'activateur de la prothrombine (thromboplastine sanguine), par l'interaction des plaquettes, du Facteur IX (PTC), du Facteur VIII (AHF), du Facteur V et du calcium, procède par une série de réactions successives. La première réaction détectable est lente et nécessite les plaquettes, le PTC et le calcium. Un produit intermédiaire ("PTC activé") est obtenu qui est débarrassé de toute contamination plaquettaire et présente une cinétique caractéristique. Les plaquettes sont également nécessaires à un stade ultérieur dans lequel elles forment probablement par l'interaction avec le "PTC activé", le AHF et le calcium un autre produit intermédiaire. Des variations de la concentration des plaquettes, de l'AHF et du PTC influencent d'une façon caractéristique la formation de la thrombine.

Zusammenfassung

Das Studium des Verhaltens gereinigter menschlicher Gerinnungsfaktoren läßt vermuten, daß die Bildung des „Prothrombinaktivators" (Blutthrombokinase) aus Plättchen, PTC (Faktor IX), AHF (Faktor VIII), Faktor V und

Calcium durch eine Reihe aufeinanderfolgender Reaktionen zustande kommt. Die früheste nachweisbare Reaktion geht langsam vor sich und benötigt Plättchen, PTC und Calcium. Es wird ein Zwischenprodukt erhalten („aktiviertes PTC"), das frei ist von Plättchenbestandteilen und das durch sein Verhalten in der Kinetik charakterisiert ist. Die Plättchen werden auch noch in einer späteren Phase der Reaktionsfolge benötigt, möglicherweise für die Bildung eines weiteren Zwischenproduktes, zusammen mit aktiviertem PTC, AHF und Calcium. Variationen in der Konzentration der Plättchen, des AHF und PTC beeinflussen die Kinetik der Thrombinbildung in charakteristischer Weise.

References

(1) Bergsagel, D. E.: The Role of Calcium in the Activation of Christmas Factor. Brit. J. Haematol. 7: 199 (1955).
(2) Bergsagel, D. E. and Biggs, R.: The Christmas Factor. Rev. Hémat. 10: 354 (1955).
(3) Bergsagel, D. E. and Hougie, C.: Intermediate Stages in the Formation of Blood Thromboplastin. Brit. J. Haematol. 2: 113 (1956).
(4) Biggs, R., Douglas, A. S. and Macfarlane, R. G.: The Initial Stages of Blood Coagulation. J. Physiol. (Lond.) 122: 538 (1953).
(5) Nour-Eldin, F. and Wilkinson, J. F.: The Isolation of Antihaemophilic Globulin from Brain Tissue. Brit. J. Haemat. 2: 433 (1956).
(6) Surgenor, D. M., Alexander, B., Schmid, K., Goldstein, J.: A system for the separation of the protein components of human plasma. II. The components of clotting mechanism. J. Phys. Coll. Chem. 55: 94—101 (1951).
(7) Surgenor, D. M. and Steele, B. B.: Platelet-Antihemophilic Factor Interactions. Federation Proceedings 17: 1262 (1958).
(8) Surgenor, D. M., Walton, K., Katz, J. H. and Steele, B. B.: In preparation.
(9) Surgenor, D. M. and Wilson, N. A.: In preparation.
(10) Tullis, J. L.: Preservation of Platelets. Amer. J. Med. Sci. 226: 191 (1953).
(11) Tullis, J. L., Surgenor, D. M. and Baudanza, P.: Preserved Platelets, Their Preparation, Storage and Clinical Use. Blood, in press.
(12) Tullis, J. L., Surgenor, D. M., Tinch, R. J., d'Hont, M., Gilchrist, F. L., Driscoll, S. and Batchelor, W. H.: The Principle of Closed System Centrifugation. Science 124: 792 (1956).
(13) Wallach, D. F. H., Surgenor, D. M. and Steele, B. B.: Calcium-Lipid Complexes in Human Platelets. Blood 13: 589 (1958).
(14) White, S. G., Aggeler, P. M. and Glendening, M. B.: Plasma thromboplastin component (PTC), a hitherto unrecognized blood coagulation factor; case report of PTC deficiency. Blood 8: 101 (1953).