The Preparation of Plasma Thromboplastin Antecedent (PTA) and its Assay with Purified Clotting Components

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IT HAS BEEN apparent from its inception that the thromboplastin generation test[1,2] is more complicated than the simple interaction, in the presence of calcium, of three factors whose names are given to the reactants of the test: platelets, antihemophilic factor and Christmas factor (plasma thromboplastin component, PTC). The platelet reactant contributes at least two factors active in this test: a protein-linked phospholipid, and accelerator globulin (AcG), the latter probably having been adsorbed from plasma. The antihemophilic factor, supplied as aluminum hydroxide- or barium sulfate-adsorbed plasma, is accompanied by at least three other factors: accelerator globulin (AcG), plasma thromboplastin antecedent (PTA)[3] and Hageman factor.[4] It may also contain inhibitors. The Christmas factor (PTC), supplied as diluted serum, is accompanied by PTA, Stuart factor,[5] Hageman factor, antithrombin, antithromboplastin and variable amounts of prothrombin. These complexities severely limit the interpretation and understanding of the reactions that are taking place.

We wish to describe a system for the assay of certain coagulation factors which replaces the complex reagents of the thromboplastin generation test with purified* fractions, the concentrations of which can be controlled. We have found that plasma thromboplastin antecedent must be incorporated in the thromboplastin generation system, and this requirement established a laboratory assay for this factor that is not dependent on the use of PTA-deficient plasma. We have employed this same test system for the semi-quantitative assay of each of its components.

MATERIAL AND METHODS

Materials

Platelet-substitute (P-S).—A lipid extract of calf brain, prepared by the method of Bell and Alton,[6] was substituted for platelets. Acetone-dried calf brain was extracted with chloroform, and the chloroform was evaporated from the extract. The residue was emulsified in 0.9 per cent saline. This material may be prepared in quantity; it is stable when kept frozen, and its performance in the assay is comparable to that of platelets. It restored normal prothrombin consumption to platelet-poor normal plasma when the latter was recalcified. Use of the platelet-substitute eliminates the difficulties of interpretation which arise when platelets are used.

Accelerator globulin (AcG).—Bovine serum was used as a source of accelerator globulin

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*The term "purified" as used in this manuscript refers to components which have been isolated and freed of other known clotting components.

because it is readily obtained and its AcG content and stability are greater than that in human serum. Bovine blood, collected without an anticoagulant, was allowed to stand at room temperature for 4 hours. Without disturbing the clot, the serum was decanted, treated with commercial barium carbonate (Bakers, 40 mg./ml.) and centrifuged. The supernatant serum was then adsorbed with freshly precipitated barium sulfate. The barium sulfate was prepared as follows: for each 100 ml. of serum to be adsorbed, 4.0 ml. of 1 M $BaCl_2$ was mixed with 4.0 ml. of 1 M Na_2SO_4. The precipitate of barium sulfate which formed was diluted with 80 ml. of distilled water and centrifuged.

The barium sulfate precipitate, with adsorbed AcG, was washed once with 200 ml. of 0.9 per cent saline and eluted with 50 ml. of 2 per cent citrate solution (2 Gm. $Na_3C_6H_5O_7.2\ H_2O$ per 100 ml. distilled water). The eluate, containing AcG, was dispensed into small vials and frozen. When kept at −20 C., it remained active for at least one year (based on its ability to normalize the prothrombin time in aged, AcG-free, oxalated, normal human plasma). The preparation was free of thrombin and prothrombin.

Antihemophilic globulin (AHG).—Fraction I was separated from fresh normal human plasma by the method of Cohn et al.[7] (method 6), within 24 hours of the time the blood was collected. Each kilogram of fraction I paste was resuspended in 12 L. of 0.025 M sodium citrate buffer (pH 6.3), filtered under sterile conditions, dispensed in vials and dried from the frozen state. For use, one vial, containing 200 mg. of fraction I protein, was reconstituted with 0.9 per cent saline, heated at 56 C. for 5 minutes, quickly cooled and the coagulated fibrinogen removed by centrifugation. The supernatant liquid, containing AHG, was dispensed in 1 ml. aliquots and stored at −20 C. until needed.

Plasma thromboplastin component (PTC).—Bovine serum was adsorbed with commercial barium sulfate (Baker, C. P.: 100 mg./ml. serum). The barium sulfate was washed twice with two-thirds serum volume of 0.01 M sodium oxalate and eluted with one-tenth serum volume of 2 per cent sodium citrate solution. The eluate was dialyzed against 0.9 per cent saline at 2 to 4 C. The dialyzed eluate was centrifuged and the supernatant solution was dispensed in small aliquots and stored frozen at −20 C. The method is so similar to those published for serum prothrombin conversion accelerator[8] and PTC[9] that the preparation can be expected to contain both components.

Plasma thromboplastin antecedent (PTA).—Fraction IV-1 was separated from normal human plasma by the method of Cohn et al.[7] (method 6). One hundred Gm. of fraction IV-1 paste was washed twice with 1 L. of distilled water at 0 C. Each time, a uniform suspension was made in a homogenizer or in a colloid mill. The washings were discarded. The washed paste was resuspended in 500 ml. of 0.15 ionic strength acetate buffer, pH 5.2. The acetate extract was recovered by centrifugation and was diluted to ionic strength 0.05 by the addition of 1 L. of distilled water. The precipitate which formed was removed by centrifugation and discarded. The supernatant liquid was further diluted to ionic strength 0.015 by adding 3500 ml. distilled water. The precipitate which formed contained PTA, and was recovered by centrifugation. The precipitate was resuspended in 100 ml. of 0.3 per cent saline and was then adjusted to pH 2.9 by the addition of 1 N hydrochloric acid. The solution at pH 2.9 was heated in a 37 C. water bath for two hours. A small precipitate formed; it was removed by centrifugation. The supernatant fluid was adjusted to pH 7.3 to 7.4 by the addition of 1 N sodium hydroxide. The PTA was stored frozen (but will keep for weeks in a refrigerated bath at 0 C.). The preparation is apparently free from PTC, AcG, AHG, Stuart factor, prothrombin and thrombin. Data supporting the identity of this preparation appear in a later part of this report.

Substrate plasma.—Fresh normal human plasma, obtained from blood drawn in acid-citrate-dextrose anticoagulant, was dispensed in 2 ml. aliquots and kept frozen at −20 C. until needed. For use, it was thawed quickly in a water bath at 37 C., after which it was kept in a container with crushed ice. Normal oxalated or citrated plasma served equally as well as ACD plasma. The platelet content of the substrate does not affect the test.

Calcium chloride.—0.020 M solution in distilled water.

Method of Assay

Modified thromboplastin generation test mixture A.—0.15 ml. platelet-substitute (diluted 1 to 25 in 0.9 per cent saline), 0.30 ml. barium sulfate-adsorbed plasma (1 to 5 in saline), and 0.30 ml. serum (1 to 10 in saline), all made to 0.9 ml. with 0.9 per cent saline.

Thromboplastin generation test mixture with purified reagents B.—The amount of each factor required depends upon the potency of the preparation and may vary from lot to lot. The following amounts were typical: 0.15 ml. platelet-substitute (diluted 1 to 25 in 0.9 per cent saline), 0.25 ml. AHG (undiluted), 0.10 ml. AcG (undiluted), 0.15 ml. PTC (diluted 1 to 5 in saline), 0.05 ml. PTA (undiluted), and 0.20 ml. saline, 0.9 per cent, to make total volume to 0.9 ml.

Procedure for both test mixture A and test mixture B.—The reagents were kept on ice until mixed. The thromboplastic test mixture, and six tubes of substrate plasma (0.1 ml. in 11 x 75 mm. glass tubes), were placed in a 37 C. water bath. After allowing two minutes for the mixture to warm to 37 C., 1.0 ml. of 0.02 M $CaCl_2$, also at 37 C., was added to it. At intervals of 1, 3, 5, 7, 9 and 11 minutes after addition of calcium, a 0.3 ml. sample of the recalcified mixture was withdrawn and quickly blown into a tube of substrate plasma.* Sufficient calcium was carried over in the sample that it was not necessary to recalcify the substrate plasma simultaneously, as is necessary in the Biggs and Douglas test. The substrate tube was left at 37 C. until shortly before the expected end point; it was then tilted gently and repeatedly until the liquid was observed to gel.

When the reagents were present in optimal amounts, a minimum clotting time of 9 ± 2 seconds was attained. Omission of any single component resulted in significantly longer clotting times.

Results and Discussion

Demonstration of PTA Effect

The requirement for PTA in the thromboplastin generation test was readily demonstrated with the aid of purified reagents. Since PTA is present in both barium sulfate-adsorbed plasma and in serum, according to studies by Rosenthal,[3] both of these reagents had to be replaced before the effect of PTA could be shown. The barium sulfate-adsorbed plasma was replaced with our AHG and AcG preparations. This considerably simplified the system, since the purified components were free of PTA, traces of prothrombin and inhibitors normally present in adsorbed plasma. The system was simplified further by replacing platelets with the platelet-substitute.

Figure 1 shows data demonstrating the presence of PTA in serum, and its requirement in the thromboplastin generation test. Rapid development of thromboplastic activity was observed with platelet-substitute, AHG, AcG and normal serum (curve 1). Adsorption of the serum with barium sulfate, thus removing PTC, resulted in marked loss of activity (curve 2). Substitution of the barium-adsorbed material (the PTC preparation) for serum resulted in even greater loss of activity (curve 3). Combination of PTC and barium sulfate-adsorbed serum (curve 4) restored the activity to that of the original system. This demonstrated that the serum contained two

*This method of recalcification, which eliminates the necessity of separately recalcifying the substrate plasma, was observed at Children's Hospital, Detroit, Mich., through the courtesy of Drs. W. W. Zuelzer and J. Rutzky and Miss Ruth Evans.

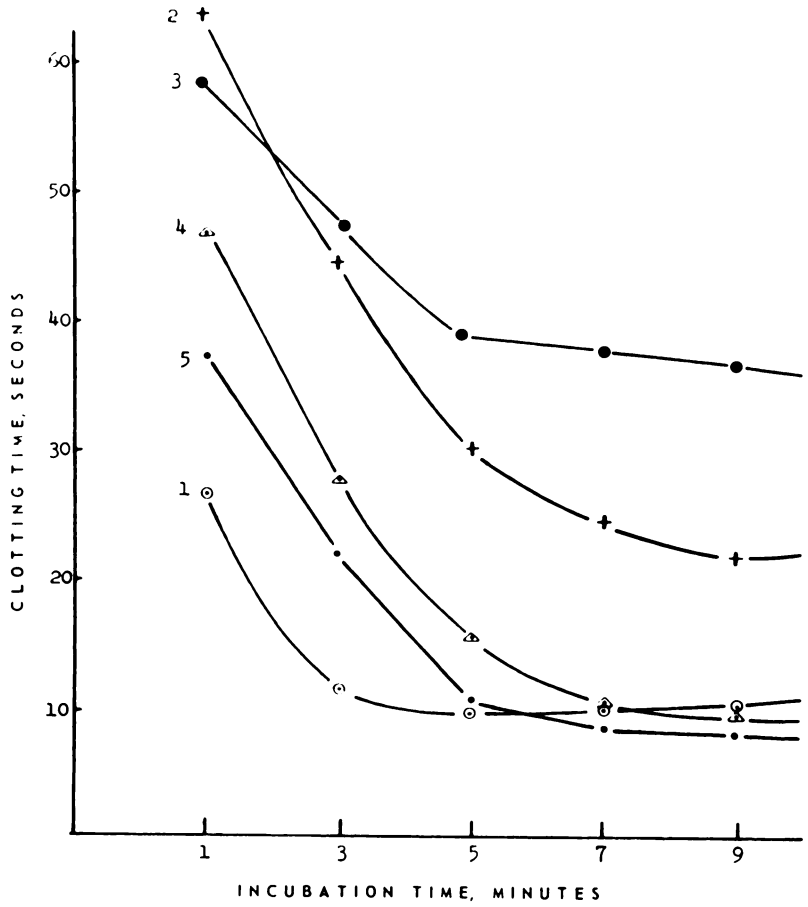

Fig. 1.—Demonstration of PTA effect in serum. ***All curves:*** **P-S, AHG, AcG constant. Incubation mixture made to 0.9 ml. with saline. Recalcified with 1.0 ml. $CaCl_2$. Assayed at intervals by transfer of 0.3 ml. to 0.1 ml. substrate plasma.**
Curve 1, **with normal serum, 0.15 ml. of 1 to 5 dilution.**
Curve 2, **with $BaSO_4$-adsorbed normal serum, 0.15 ml. of 1 to 5 dilution.**
Curve 3, **with purified PTC, 0.15 ml. of 1 to 5 dilution.**
Curve 4, **with $BaSO_4$-adsorbed normal serum plus PTC.**
Curve 5, **with purified PTC plus purified PTA.**

factors: one adsorbable and the other nonadsorbable on barium sulfate. The combination of PTC and purified PTA (curve 5) was also fully active, which showed that PTA could be substituted for barium sulfate-adsorbed serum. Thus we have shown that both PTC and PTA are required to replace normal serum in the thromboplastin generation test.

Identification of PTA Using PTA-deficient Plasma

The identity of purified PTA was confirmed on the basis of its corrective effect in the thromboplastin generation test, with the use of PTA-deficient

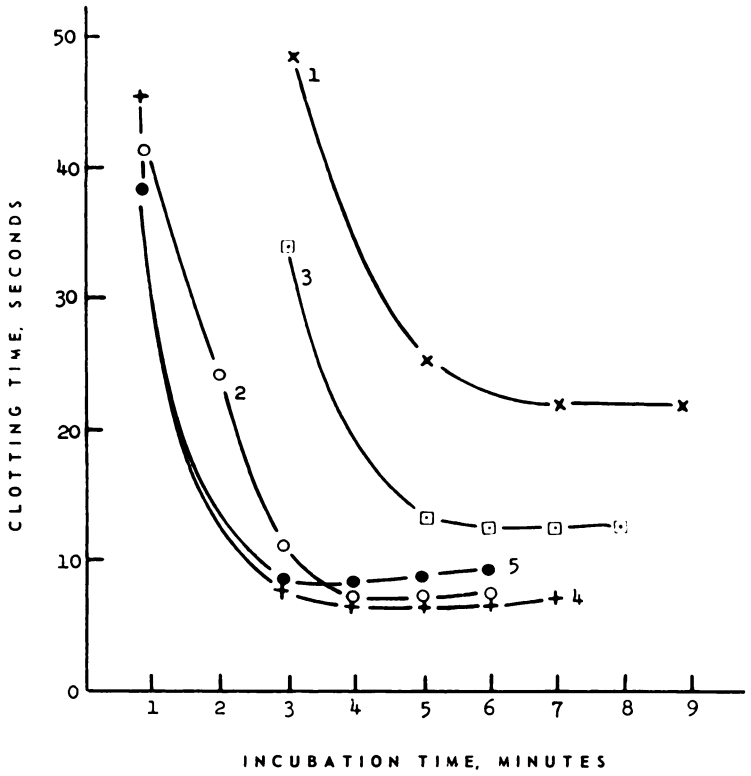

Fig. 2.—Thromboplastin generation in PTA deficiency and its correction by PTA. ***All curves:*** **P-S, 0.15 ml., in place of platelets. $BaSO_4$ plasma, 0.3 ml. of 1 to 5 dilution. Serum, 0.3 ml. of 1 to 10 dilution. Incubation mixture made to 0.9 ml. with saline. Recalcified with 1.0 ml. $CaCl_2$. Assayed at intervals by transfer of 0.3 ml. to 0.1 ml. substrate plasma.**

Curve 1, **patient's $BaSO_4$ plasma plus patient's serum.**

Curve 2, **normal $BaSO_4$ plasma plus normal serum.**

Curve 3, **normal $BaSO_4$ plasma plus patient's serum.**

Curve 4, **patient's $BaSO_4$ plasma plus normal serum.**

Curve 5, **patient's $BaSO_4$ plasma plus patient's serum plus PTA.**

patient's plasma and serum* (fig. 2). With the patient's barium sulfate-adsorbed plasma and serum, abnormal thromboplastin generation was observed (curve 1), as compared to the corresponding reagents from a normal individual (curve 2). The deficiency was partially corrected by substituting normal barium sulfate-adsorbed plasma for that of the patient (curve 3), and was fully corrected by substituting normal serum for the patient's serum (curve 4). The abnormal thromboplastin generation with patient's barium

*The PTA-deficient plasma and serum were obtained from a patient whose physician requested our assistance in the study of a bleeding tendency. The diagnosis of PTA deficiency in this patient has been confirmed by Dr. R. L. Rosenthal,[10] who also confirmed the corrective effect of our PTA preparation in vitro in the plasma of one of his own PTA-deficient patients.

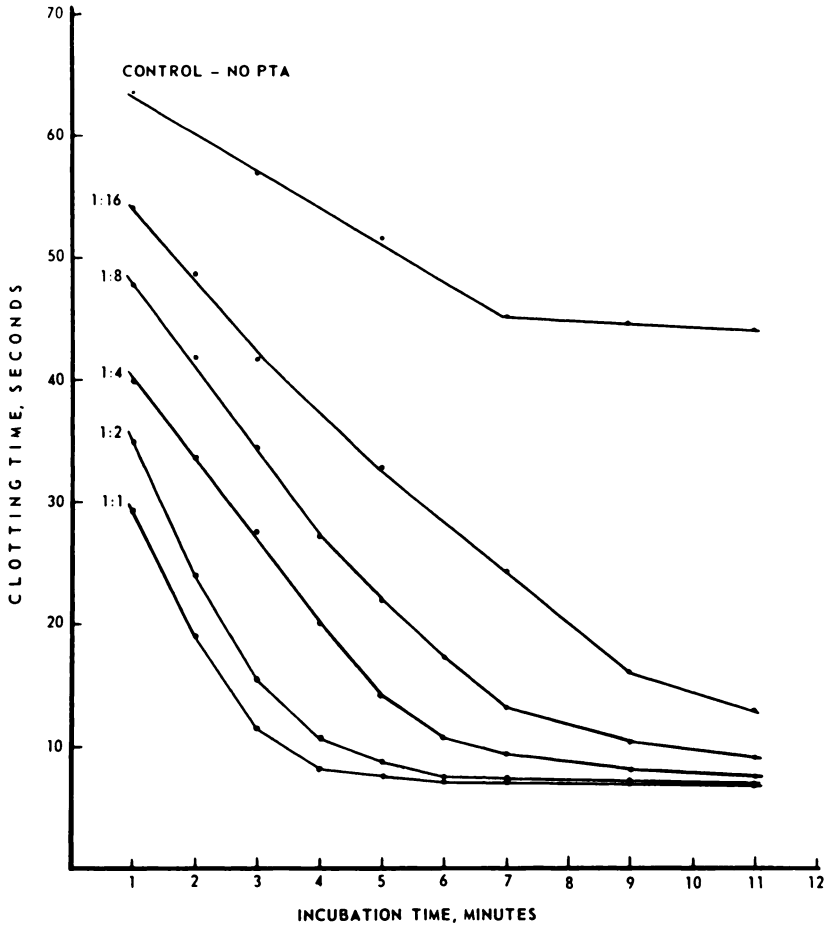

Fig. 3.—Thromboplastin generation with purified components and varying quantities of purified PTA. *All curves:* P-S, AHG, AcG, PTC constant. Variable PTA, and incubation mixture made to 0.9 ml. with saline. Recalcified with 1.0 ml. $CaCl_2$. Assayed at intervals by transfer of 0.3 ml. to 0.1 ml. substrate plasma. *(PTA,* dilutions of stock solution in saline.)

sulfate-adsorbed plasma and serum was corrected also by the addition of purified PTA (curve 5). The difference between curves 1 and 5 is the result of the presence of purified PTA in the thromboplastin generation mixture of the latter.

Assay of PTA and other Factors with Purified Reagents in Vitro

From the standpoint of convenience and practicality, it is desirable that an in vitro assay would *not* require the use of plasma or serum deficient in a particular factor. Such an assay is obtained when purified reagents are used throughout (see METHODS, Thromboplastin Generation Test Mixture With Purified Reagents). Here, the test system is dependent on the presence of all components, and each component can be varied at will with the others

held constant. For each level of the factor studied, a thromboplastin generation curve was determined (plotting clotting time against incubation time). Thus, a family of curves was constructed by using several dilutions of a reference lot for each coagulation factor.

One such family of curves for PTA is shown in figure 3. The other components of the system (P-S, AHG, AcG, PTC) were held at constant, nonlimiting levels. Various levels of PTA, from 1:1 (no dilution) to a 1:16 dilution, are shown, compared to the control curve at the top, which shows the activity in the absence of PTA. PTA, purified from fraction IV-1 of plasma, was preferred to barium sulfate-adsorbed serum as a source of PTA, because the former does not contain serum factors such as antithromboplastin.

We have not attempted to define a unit of PTA activity, pending further study of the test system. At present, it is preferable to assay the PTA content of a test specimen by choosing a dilution of the specimen which will fall on or between two levels of a reference lot of PTA, assayed on the same day as the unknown.

The test mixture with purified reagents, as described, does not require the addition of Stuart factor or Hageman factor as specific components. Some Stuart factor is probably supplied by the PTC preparation, since the PTC partially corrects the abnormal thromboplastin generation in a system containing platelet-substitute, barium sulfate-adsorbed normal plasma and Stuart-deficient serum.*

The possible role of Hageman factor in this system is not clear. Published properties of Hageman factor[4,11] are similar to those of PTA, and both factors are reported to be activated by contact with wettable glass.[12,13] Our preparation of PTA shortens the recalcified clotting time of silicone-collected plasma in silicone-coated glass. Whether this is a reflection of its PTA activity or of its contamination with Hageman factor will have to be determined. It also remains to be determined whether or not Hageman factor contributes activity to the system which might be mistaken for PTA activity.

Summary

By replacing the three crude reagents commonly used in thromboplastin generation tests—washed platelets, barium sulfate- or alumina-adsorbed plasma, and serum—with purified clotting factors, many variables and uncertainties were eliminated. It was demonstrated that plasma thromboplastin antecedent (PTA) was required for the generation of thromboplastic activity.

A method was developed for the preparation of purified PTA from fraction IV-1 of human plasma. Its identity was established by its ability to correct, in vitro, the defect in the plasma of a PTA-deficient patient. Thus, further evidence in support of the belief that PTA is a discrete component, essential

*Stuart factor-deficient serum was received through the courtesy of Charles L. Johnston, M.D., School of Medicine, The University of North Carolina, Chapel Hill, N. C.

for blood coagulation, was obtained. More specifically, it was found to be essential in the generation of thromboplastic activity in plasma.

A test system for thromboplastin generation was described which was used to assay PTA in vitro and which did not require the use of PTA-deficient plasma or serum. Omission of any one of the components of this system resulted in a marked loss of thromboplastic activity; restoration of activity was proportional to the amount of the component that was added. Thus, with this system of purified components, it was possible to assay any one of them without the use of, or requirement for, plasma or serum specimens from patients with specific coagulation deficiencies.

Summario in Interlingua

Per introducer purificate factores coagulatori in loco del tres crude reagentes que es communmente usate in tests de generation de thromboplastina —i.e. plachettas eluite, plasma adsorbite a sulfato de barium o alumina, e sero—multe variabiles e multe incertitudes esseva eliminate. Esseva demonstrate que antecedente de thromboplastina del plasma (ATP) es indispensabile in le generation de activitate thromboplastic.

Esseva disveloppate un methodo pro le preparation de ATP purificate ab fraction IV–1 de plasma human. Su identitate esseva establite per su capacitate de corriger in vitro le defecto in le plasma de patientes deficiente in ATP. Assi un nove supporto esseva obtenite pro le conception que ATP es un componente discrete que es essential in le coagulation de sanguine. Plus specificamente, il esseva trovate que ATP es essential in le generation de activitate thromboplastic in le plasma.

Es describite un systema pro le effectuation de tests del generation de thromboplastina. Illo esseva usate pro le essayage de ATP in vitro. Illo es distinguite per le facto que illo non require le uso de plasma o sero deficiente in ATP. Le omission de non importa le qual del componentes de iste systema resultava in un perdita marcate de activitate thromboplastic. Le restauration del activitate esseva proportional al quantitate del componente que esseva re-addite. Assi il esseva possibile per medio de iste systema de purificate componentes de effectuar tests pro le presentia de non importa le qual de ille componentes sin le uso e sin e requirimento de specimens ab patientes con specific defectos de coagulation.

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