The Identification and Synthesis of Activated Plasma Thromboplastin Component (PTC')

By Sandra Schiffman, Samuel I. Rapaport and Mary Jane Patch

As the number of known intrinsic clotting factors has increased, it has become clear that the final reaction, the conversion of fibrinogen to fibrin by thrombin, must result from a series of preliminary reactions, each probably involving only two clotting factors. This means that each clotting factor must circulate either as a precursor or as a highly specific molecule which only reacts with another factor which circulates as a precursor. The literature on plasma thromboplastin component (PTC, factor IX, Christmas factor) contains evidence that PTC belongs to the former category, i.e., that PTC is a molecule which is activated during intrinsic clotting, in vitro.19

We report herein the identification of an activated form of PTC (PTC') in barium sulfate eluates of citrated plasma. Data will also be presented demonstrating the synthesis of PTC' from partially purified reagents. It will be shown (1) that the only clotting factors required are native PTC, the substrate, and activated plasma thromboplastin antecedent (PTA', activated factor XI), the enzyme, and (2) that ionic calcium accelerates but is not otherwise necessary for the reaction.

Materials

Intact normal plasma. Venous blood was drawn through a Monocote E (Armour Laboratories)-coated needle into a silicone (G.E. SC-87, Dri-film)-coated syringe and mixed with anticoagulant in a ratio of 9 parts of blood to 1 part of anticoagulant. The anticoagulant was either 0.1 M disodium oxalate or a citrate solution which was 0.06 M in trisodium citrate and 0.04 M in citric acid. The blood was centrifuged in plastic tubes for 10 minutes at 12,500 g at 3 C. to obtain the supernatant platelet-poor plasma. Plasmas were either used fresh or frozen in small aliquots in tightly capped, 1.2 ml. plastic vials (Polyvial, Olympic Plastics Company, Inc., Los Angeles) at −20 C. Frozen plasma was always thawed in water at 37 C. for a minimum of 3 minutes. Plasma prepared in this fashion is called intact plasma because it has not contacted an activating surface such as glass.

Specific deficiency plasmas. Intact plasmas deficient in Hageman factor (HF, factor XII), plasma thromboplastin antecedent (PTA, factor XI), anti-hemophilic globulin (AHG, factor VIII), PTC, or proaccelerin (factor V) were obtained by the technic described above from patients with hereditary deficiencies of these clotting factors. These patients have been described elsewhere.10 Intact Stuart factor (factor X) deficiency plasma from Mrs. L. was obtained through the courtesy of Miss Lorraine Gonyea of the Depart-
ment of Laboratory Medicine, University of Minnesota. Proconvertin (factor VII) deficiency plasma, which had been lyophilized in glass ampules, was obtained from Dr. Charles Hall of the Albany V. A. Hospital from his patient Mr. G. D., and from Dr. P. A. Owren of Oslo, Norway, from his patient Mr. H. This plasma was reconstituted in distilled water just prior to use and its pH adjusted to 7.3 with 0.1 N HCl.

**Exhausted plasma** was prepared by a technic, described elsewhere, in which normal, intact plasma was allowed to flow through a column packed with diatomaceous earth powder (Dicalite 4200, Great Lakes Carbon Co., Waltheria, Calif.). A preparation suitable for use as the substrate for the PTA' assay (vide infra) contained the following clotting factor activities: HF, 0 per cent; PTA, 0.5 per cent; PTC, 35 per cent; AHG, 30 per cent; Stuart factor, 120 per cent; proaccelerin, 42 per cent; proconvertin, 100 per cent; and prothrombin, 100 per cent.

**PTA' reagent.** The Dicalite 4200 column used to prepare exhausted plasma was washed in physiologic saline and eluted with 7 per cent sodium chloride solution. The procedure has been described in detail elsewhere under the heading, preparation of activation product (AP). As will be discussed later, it now appears that activation product is activated PTA (PTA'); therefore, the term PTA' will be used instead of the term activation product in this paper.

**Artificial exhausted serum.** To 30 ml. of exhausted plasma were added 2 ml. of thromboplastin and 1.1 ml. of 1 M CaCl₂. Ten minutes after clotting the clot was removed by centrifugation at 12,500 g. The resultant "serum" was centrifuged 2 times at 39,000 rpm for 1 hour in a Spinco Model L ultracentrifuge to sediment the thromboplastin. The supernatant was stored in plastic vials at −20°C for use as a starting material in the preparation of a PTC reagent poor in prothrombin.

**Serum for use in the thromboplastin generation test** was made by allowing blood to clot in a glass tube. After standing for 2 hours at 37°C on the clot, the serum was separated by centrifugation. To each ml. of serum, 0.2 ml. of the citrate anticoagulant was added and the mixture allowed to stand for 2 hours more at 37°C. The serum was then either used immediately or stored frozen in plastic vials at −20°C.

**Barbital buffer** is a solution of 0.028 M NaCl plus 0.125 M sodium barbital, total ionic strength 0.15. The pH is adjusted to 7.25 with 0.1 N HCl.

**Column buffer 0.693 M** is a solution of 0.02 M sodium barbital and 0.673 M NaCl. The pH is adjusted to 7.1 with HCl.

**Cephalin,** an acetone-insoluble, ether-soluble extract of human brain, was prepared as described earlier. It was diluted 1/100 in barbital buffer before addition to clotting mixtures.

**Thrombin.** Bovine thrombin (Thrombin, Topical, Parke Davis & Co.), dissolved in oxalated saline, was adsorbed with BaSO₄ to remove any trace of contaminating PTC activity. The details of adsorption and storage have been described elsewhere. The units assigned to dilute thrombin solutions are approximations since they were obtained by assuming that the original adsorbed stock solution did in fact contain 1000 N.I.H. units per ml. Dilute solutions were made in barbital buffer using Siliclad (Clay-Adams Co.)-coated pipettes and plastic tubes and were kept in an ice bath.

**Fibrinogen.** The contents of one vial of bovine Fibrinogen (Warner-Chilcott) were dissolved in 2 ml. of distilled water and 1 ml. of a fluid made by adding 1 part of 0.1 M sodium citrate to 6 parts of isotonic saline. The resultant solution should contain 200 mg. per cent fibrinogen in citrated, isotonic saline.

**Human brain thromboplastin** was prepared by Owren’s technic. **DEAE-cellulose** was obtained from California Corporation for Biochemical Research, Los Angeles. Each 100 Gm. was washed with the following: 2 liters of a solution containing 0.5 N NaOH and 0.5 M NaCl, distilled water until the pH fell to 7, 2 liters of 0.1 N HCl in ethanol, and distilled water until the pH was about 0. Shortly before use...
a small amount of treated DEAE in water was equilibrated in starting buffer until it reached the desired pH.

Twelve-by-75 mm. clear polystyrene test tubes (Falcon Plastics Co., Los Angeles) were used for all clotting tests. This prevented contact activation of test mixtures and also eliminated the problem of adsorption of dilute thrombin onto glass surfaces.

METHODS

Preparation of BaSO₄ Eluates

Plasma was adsorbed with 50 mg. of BaSO₄ (Baker Chemical Co.) per ml. for 15 minutes at 0°C and centrifuged for 10 minutes at 12,500 g. The precipitate was washed 3 times for 5 minutes with double the original plasma volume of cold physiologic saline. The BaSO₄ cake was then eluted by stirring for 15 minutes at room temperature with one-half of the original plasma volume of 5 per cent trisodium citrate in saline (5 Gm. trisodium citrate plus sufficient physiologic saline to make 100 ml.). The BaSO₄ was removed by centrifugation at 12,500 g for 10 minutes and the supernatant was dialyzed overnight against 2 large volumes of saline. Eluates were then either frozen at −20°C or allowed to age for several days at room temperature or 3°C. All steps were carried out in plastic vessels.

DEAE-cellulose Chromatography

Ion exchange chromatography was carried out in a cold room. A lucite column with an ashless filter paper support was used to prevent contact activation during chromatography. A salt concentration gradient at pH 7.1 was produced by a closed system with one reservoir and a mixer flask. The gradient, ranging from 0.05 M to 0.364 M, was formed by placing 0.05 M column buffer in the mixer flask and 0.693 M column buffer in the reservoir. Initially, the reservoir buffer volume was always two-thirds of the volume of the mixer buffer. Constant flow rate of about 1.5 ml./min. was maintained by a Sigma motor Finger Pump placed between the mixer flask and the column. The column was packed with DEAE washed in 0.05 M column buffer; the starting material was dialyzed against the same buffer using 100 ml. buffer per ml. of starting material. The following conditions were employed for the chromatography of 5 ml. of starting material: a column of diameter 1.2 cm. and height 40 cm., 150 ml. of 0.05 M column buffer in the mixer flask and 100 ml. of 0.693 M column buffer in the reservoir. Effluent fractions were collected in plastic tubes in a volume equal to that of the starting material and were dialyzed at 3°C against 2 large volumes of saline. Protein was estimated by measuring absorbency at 280 m. No attempt was made to concentrate the fractions.

Preparation of Partially Purified PTC

Intact plasma was chromatographed as described above. PTA elutes at 0.05 M (it is not bound to the resin), and HF at about 0.18 M. PTC, prothrombin, proconvertin, and Stuart factor were found to elute at about 0.3 M. About 50 per cent of the original plasma PTC activity could be recovered. The tube containing the highest PTC concentration showed a 5 to 20-fold purification based upon protein concentration measured as absorbancy at 280 m. The contents of the two to three tubes with the greatest PTC activity were pooled and stored at −20°C in plastic vials.

Clotting Factor Assays

1. Intact partial thromboplastin times (intact PTT). Aliquots of 0.1 ml. of intact normal plasma, cephalin diluted 1/100, and test reagent were incubated together for 3 minutes at 37°C in a clear plastic clotting tube. Then 0.1 ml. of 30 mM CaCl₂ was added and the clotting time noted.

2. PTA' assay. This assay has been described in detail earlier under the name preformed activation product assay. In brief, 0.1 ml. of exhausted plasma and 0.1 ml. of cephalin
1/100 were incubated in a plastic clotting tube at 37 C. for 3 minutes. Then, in rapid succession, 0.1 ml. of prewarmed 30 mM CaCl₂ and 0.1 ml. of test substance were added and the clotting time measured from the addition of the test substance.

3. PTC assays. Our PTC assays measured the ability of a test substance to correct the prolonged PTT of PTC deficiency plasma (PTC₉₅). Several assay technics were devised:

   a. The contact PTC assay was designed to provide maximum PTA' generation in the substrate by adding kaolin powder to the test mixture. To 0.1 ml. of a suspension of kaolin 8 mg./ml. in cephalin 1/100, were added 0.1 ml. of PTC₉₅ and 0.1 ml. of test substance. After 8 minutes at 37 C., 0.1 ml. of 40 mM CaCl₂ was added and the clotting time noted. The blank time was 95-115 seconds; a 1/5 dilution of normal plasma clotted in about 50 seconds. A plot of concentration of a normal reference plasma against clotting time gave a straight line on log-log paper from which activity was converted to per cent. This contact assay was used primarily to measure unactivated or native PTC, although, of course, it is also sensitive to PTC' or to other later clotting intermediates.

   b. The intact PTC assay was designed to prevent the generation of PTA' activity in the assay mixture. To 0.1 ml. of cephalin 1/100 in a plastic tube were added 0.1 ml. of PTC₉₅ and 0.1 ml. of test substance. Siliclad-coated pipettes were used. After 3 minutes at 37 C., the mixture was recalcified with 40 mM CaCl₂ and the clotting time noted. The blank time was about 140 seconds. An active undiluted BaSO₄ eluate clotted in about 7 minutes. Active BaSO₄ eluates (vide infra) clotted in 30 to 60 seconds. Thus, this assay appears to be relatively insensitive to native PTC but highly sensitive either to the combination of PTA' and PTC, or, as will be shown, to PTC'.

   c. PTC assays with thrombin. Some of our test reagents were contaminated with traces of thrombin capable of clotting fibrinogen in from 15 minutes to several hours. Since traces of thrombin shorten the PTT by activating AHG and proaccelerin, traces of thrombin in a test reagent will give erroneously high values in quantitative assays for PTC based upon this technique. To prevent this, a known concentration of thrombin, exceeding that present in the test reagents, was added to the substrate mixture to attempt to standardize this effect. As can be seen from the blank times given below, this added thrombin did not substitute for the effect of PTC, itself, in the assays.

   The contact PTC assay with thrombin was performed as follows: to 0.1 ml. of kaolin 8 mg./ml. in cephalin 1/100 were added 0.1 ml. of PTC₉₅, 0.1 ml. of 0.1 U./ml. thrombin, and 0.1 ml. of test reagent. Exactly 3 minutes later, 0.1 ml. of 40 mM CaCl₂ was added and the clotting time noted. Blank time was 120 seconds; a 1/5 dilution of normal plasma clotted in about 40 seconds.

   The intact PTC assay with thrombin was carried out as follows: to 0.1 ml. of cephalin 1/100 in a plastic clotting tube were added 0.1 ml. of PTC₉₅, and 0.1 ml. of 0.1 U./ml. of thrombin. Exactly 3 minutes later, 0.1 ml. of 40 mM CaCl₂ was added followed immediately by 0.1 ml. of the test substance. Clotting time was measured from the addition of the test substance. Blank time ranged from 5 to 8 minutes. A 1/5 dilution of intact normal plasma clotted in about 140 seconds. An active undiluted BaSO₄ eluate clotted in about 15 seconds.

   d. The PTC' generation assay was designed to measure changes in activity on incubation of PTA' and PTC with calcium. PTA' reagent, PTC reagent, and 0.315 M CaCl₂ were incubated in a ratio of 1:1:0.1. At specific intervals, 0.1 ml. of this mixture was subsampled into a second mixture containing 0.05 ml. of cephalin 1/50, 0.1 ml. of PTC₉₅, and 0.05 ml. of 0.4 U./ml. thrombin, and the clotting time was noted. This second mixture incubated for exactly 3 minutes at 37 C. before the aliquot from the first mixture was added to it. All procedures were carried out in plastic tubes.

   In a few experiments PTC activity was also evaluated in a thromboplastin generation test system. The incubation mixture consisted of 0.2 ml. aliquots of: Al(OH)₃ adsorbed normal plasma 1/5 in barbital buffer, PTC deficiency serum 1/10 in barbital buffer, cephalin 1/100, test reagent, and 30 mM CaCl₂. At specific intervals, 0.1 ml. of 20 mM CaCl₂ and 0.1 ml.
of the above incubation mixture were added to 0.1 ml. of prewarmed normal plasma and the clotting time noted.

4. Thrombin activity was measured by adding 0.1 ml. of test substance to 0.2 ml. of fibrinogen prewarmed to 37 C. and noting the clotting time. Siliclad-coated pipettes and plastic clotting tubes were used.

5. Other assays. HF was assayed as follows: to 0.1 ml. of a 2.5 mg./ml. kaolin suspension in cephalin 1/100 was added 0.1 ml. of test substance, followed 30 seconds later by 0.1 ml. of HF deficiency plasma. After 7 minutes incubation at 37 C., the mixture was recalcified with 30 mM CaCl₂ and the clotting time noted. AHG was measured by incubating 0.1 ml. of a suspension of kaolin 20 mg./ml. in cephalin 1/100, 0.1 ml. of AHG deficiency plasma, and 0.1 ml. of test substance for 3 minutes at 37 C. and then recalcifying with 0.1 ml. of 40 mM CaCl₂. Detailed conditions for the determination of PTA, proaccelerin and proconvertin have been described elsewhere. Prothrombin was measured by a one-stage venom cephalin technic to which a serum reagent (Hjort's "proconvertin reagent") was added to supply Stuart factor. Stuart factor was measured by a slight modification of Hougie's technic.

All percentages are relative to a single normal plasma used throughout these studies as the standard reference.

RESULTS

A. Identification of PTC' in BaSO₄ Eluates of Citrated Plasma

Eluates of barium sulfate exposed to intact, normal, citrated plasma developed powerful procoagulant activity capable of shortening the intact PTT from times of greater than 100 seconds to times of 10 to 30 seconds. The same effect was obtained with eluates prepared from plasmas deficient in AHG, in proaccelerin, in Stuart factor, and in proconvertin.

This procoagulant activity resembled PTA' in that it was demonstrable in the absence of an activating surface; it appeared to bypass the contact activation reaction. Therefore, it was not surprising to find that eluates prepared from HF and PTA deficiency plasmas lacked activity since HF and PTA are required to generate PTA'. However, full procoagulant activity also failed to develop in eluates prepared from PTC deficiency plasma (see table 1). This meant that the activity could not simply be PTA'; it implied that the activity might stem from an activation of PTC requiring PTA'.

As the data in table 1 illustrate, fresh eluates exhibited some procoagulant activity. This increased strikingly as the eluates aged. It appeared as if whatever was responsible for the initial activity of the eluates continued to form as the eluates aged.

The eluates contained appreciable amounts of PTC, proconvertin, Stuart factor, and prothrombin, and traces of AHG and proaccelerin. In table 2, quantitative values for these activities are given for two eluates before and after aging. (Quantitative values for HF and PTA are not listed, for, as will be more apparent later, these factors cannot be measured in the presence of activated PTC.) As can be seen, the PTC activity of the eluates increased strikingly on standing. The activity of the other clotting factors remained essentially unchanged, or decreased.

It is important to stress that the increase in PTC activity illustrated in table 2 was demonstrable not only in an intact PTC assay but also in a contact PTC assay, i.e., in an assay where maximum PTA' activity is generated in the sub-
Table 1.—The Procoagulant Effect of BaSO₄ Eluates of Citrated Normal and Deficiency Plasmas before and after Aging

<table>
<thead>
<tr>
<th>Test Reagent</th>
<th>Fresh Eluate</th>
<th>Aged Eluate*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Intact PTT (sec.)</td>
<td>Intact PTT (sec.)</td>
</tr>
<tr>
<td>Eluates of:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal plasma</td>
<td>62, 61</td>
<td>26</td>
</tr>
<tr>
<td>AHG deficiency plasma</td>
<td>71, 58</td>
<td>25</td>
</tr>
<tr>
<td>Proaccelerin deficiency plasma</td>
<td>73, 74</td>
<td>28</td>
</tr>
<tr>
<td>Stuart factor deficiency plasma</td>
<td>92, 93</td>
<td>33</td>
</tr>
<tr>
<td>Proconvertin deficiency plasma</td>
<td>28, 28</td>
<td>15</td>
</tr>
<tr>
<td>Hageman factor deficiency plasma</td>
<td>157,130</td>
<td>121</td>
</tr>
<tr>
<td>PTA deficiency plasma</td>
<td>113,122</td>
<td>101</td>
</tr>
<tr>
<td>PTC deficiency plasma</td>
<td>110,120</td>
<td>73</td>
</tr>
<tr>
<td>Saline</td>
<td>110–155</td>
<td>no clot</td>
</tr>
<tr>
<td>Thrombin, 0.2 U./ml</td>
<td>49</td>
<td>4.5</td>
</tr>
</tbody>
</table>

*Allowed to stand in plastic tubes for 7 days at 3 C.

Table 2.—The Effect of Aging upon the Activity of Clotting Factors in Two BaSO₄ Eluates of Citrated Normal Plasma

<table>
<thead>
<tr>
<th>PTC</th>
<th>Contact %</th>
<th>Intact %</th>
<th>Proconvertin %</th>
<th>Stuart factor %</th>
<th>Prothrombin %</th>
<th>AHG %</th>
<th>Proaccelerin %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Fresh eluate</td>
<td>80</td>
<td>38</td>
<td>95</td>
<td>100</td>
<td>70</td>
<td>46</td>
</tr>
<tr>
<td></td>
<td>Aged eluate</td>
<td>190</td>
<td>31</td>
<td>42</td>
<td>100</td>
<td>22</td>
<td>42</td>
</tr>
<tr>
<td>2.</td>
<td>Fresh eluate</td>
<td>48</td>
<td>60*</td>
<td>95</td>
<td>215*</td>
<td>40</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>Aged eluate</td>
<td>135</td>
<td>48*</td>
<td>88*</td>
<td>46</td>
<td>8</td>
<td>17</td>
</tr>
</tbody>
</table>

*Elute tested at a 1:5 dilution.

strate plasma. Therefore, it seemed most unlikely that generation of PTA' in the eluates was causing an apparent increase in their PTC activity. Moreover, only small amounts of PTA' could have formed in the eluates, for only small amounts of HF and PTA are absorbed onto barium sulfate from citrated plasma (see table 3).

All active aged eluates were found to contain traces of thrombin insufficient to clot plasma but capable of clotting a fibrinogen solution in from about 15 to 30 minutes (see table 1). Minute amounts of thrombin accelerate intrinsic prothrombinase generation in PTT systems by activating AHG and proaccelerin. However, this phenomenon could not account for the entire procoagulant effect of the eluates. As table 1 also shows, a concentration of thrombin capable of clotting a fibrinogen solution in less than 5 minutes failed to shorten the PTT of normal plasma to the same extent as the active eluates.

Yet, the possibility remained that the increase in PTC activity on standing was an artifact resulting from contamination of the assay mixture with minute amounts of thrombin in the test reagent. Therefore, fresh and aged eluates were assayed using the contact and intact PTC assays with thrombin described above. As table 4 shows, increased PTC activity was still demonstrable.
Identification and Synthesis of PTC'

Table 3—The Effect of \( \text{BaSO}_4 \) Adsorption upon the HF and PTA Content of Three Normal Citrated Plasmas

<table>
<thead>
<tr>
<th></th>
<th>HF %</th>
<th></th>
<th></th>
<th>PTA %</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Plasma</td>
<td>175</td>
<td>175</td>
<td>140</td>
<td>115</td>
<td>125</td>
<td>125</td>
</tr>
<tr>
<td>Absorbed plasma</td>
<td>110</td>
<td>115</td>
<td>90</td>
<td>100</td>
<td>75</td>
<td>80</td>
</tr>
</tbody>
</table>

in the aged eluates. Therefore, we concluded that PTC had been activated. It appeared as if the remarkable shortening of the intact PTT produced by aged eluates stemmed from the combined effect of activated PTC and traces of thrombin.

Further evidence of activated PTC in eluates prepared from citrated plasma was obtained by comparing the results of barium sulfate adsorption of oxalated and citrated plasma. As the example in table 5 illustrates, barium sulfate removed essentially all of the PTC activity from oxalated plasma but only a part of the PTC activity from citrated plasma. Since the plasmas were handled identically, one would expect an eluate from oxalated plasma to contain more PTC activity than an eluate from citrated plasma. Yet, fresh eluates from citrated plasma consistently appeared to contain more PTC than fresh eluates from oxalated plasma. This suggested that part of the PTC in the eluate from citrated plasma had been activated during its preparation. On standing for a week or more the eluates from citrated plasma gained PTC activity whereas those from oxalated plasma lost most of their PTC activity (see table 5). Since these changes were demonstrable in assay systems providing maximum PTA' activity, they may be taken as evidence (1) that activated PTC began to form during the preparation of eluates from citrated but not from oxalated plasma, and (2) that its concentration increased with aging in eluates from citrated but not in eluates from oxalated plasma.

Direct evidence of the existence of an activated form of PTC, PTC', whose expression no longer required the presence of PTA', was obtained by column chromatography on DEAE-cellulose (see table 6). When normal intact plasma was chromatographed using the gradient described in the methods section, native PTA was eluted at 0.05 M and native PTC was eluted at about 0.3 M. Essentially no apparent PTA' activity was obtained in either region. When a PTA' reagent was chromatographed, virtually all of the coagulant activity was eluted at 0.05 M, i.e., the same elution pattern was obtained as for native PTA from intact plasma. Only a trace of coagulant activity was obtained in the PTC area. In contrast, when, on four occasions, an active barium sulfate eluate from citrated normal plasma was chromatographed, little or no PTA' activity was found in the PTA area. Rather, a coagulant activity now appeared in the PTC area which combined the coagulant effect of PTA' and PTC.

Some of the fractions of barium sulfate eluate eluted in the PTC area were still contaminated with traces of thrombin. For example, the fraction used to obtain the data shown in table 6 clotted a fibrinogen solution in 105 minutes. Therefore, as a confirmatory test, the activity of fractions from the PTC area
Table 4.—Measurement of PTC’ Activity in BaSO₄ Eluates Using Assays Containing Thrombin

<table>
<thead>
<tr>
<th></th>
<th>PTC-contact (sec.)</th>
<th>PTC-intact (sec.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Fresh eluate</td>
<td>43*</td>
<td>32</td>
</tr>
<tr>
<td>Aged eluate</td>
<td>38*</td>
<td>20</td>
</tr>
</tbody>
</table>

*Eluate tested at a 1:5 dilution.

Table 5.—The Effect of Adsorption, Elution, and Aging on the PTC Activity of Citrated and Oxalated Plasma

<table>
<thead>
<tr>
<th></th>
<th>Citrated</th>
<th>Oxalated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma</td>
<td>75</td>
<td>75</td>
</tr>
<tr>
<td>Adsorbed plasma</td>
<td>55</td>
<td>0</td>
</tr>
<tr>
<td>Fresh eluate</td>
<td>80</td>
<td>50</td>
</tr>
<tr>
<td>Aged eluate</td>
<td>190</td>
<td>1.5</td>
</tr>
</tbody>
</table>

was rechecked in the intact PTC assay with thrombin. Activated PTC was still readily demonstrable. Thus, as the data in table 7 illustrate, much shorter clotting times were obtained with a 0.3 M column fraction than with undiluted intact normal plasma. Clearly, the 0.3 M fraction had PTC activity which no longer required the contact activation reaction for its expression, i.e., it contained PTC’.

B. Synthesis of PTC’ from Partially Purified Reactants

The foregoing data fit the hypothesis that PTC’ forms by an interaction between PTA’ and PTC. Semi-purified PTA’ and PTC preparations were made to study three aspects of this interaction: (1) the role of calcium, (2) the role, if any, of other clotting factors, and (3) the kinetics of the reaction.

1. The role of calcium. The generation of PTC’ activity in the aging BaSO₄ eluates could not have required calcium; these eluates were made in citrate, a good calcium chelating agent, and were then dialyzed against large volumes of physiologic saline. To confirm that PTC’ could form in the absence of calcium, the reaction was studied further using two sources of PTA’ and PTC. In one experiment, a BaSO₄ eluate of PTC deficiency plasma (PTA’ source) was mixed with a BaSO₄ eluate of HF deficiency plasma (PTC source). The mixture, examined after standing for 8 days at 3 C., contained PTC’ activity. In a second experiment, PTA’ reagent from normal plasma was mixed with PTC made by DEAE-cellulose column chromatography of intact plasma. When this mixture was examined, after 6 days incubation at 3 C., PTC’ activity was again found. The exact time required to generate PTC’ in the absence of calcium was not determined; in both experiments the reaction had already taken place by the time the mixtures were examined. These data are shown in table 8.

However, as the data shown in the bottom part of table 8 illustrate, calcium is required if the reaction between PTA’ and PTC is to occur rapidly. Incubation mixtures of PTA’ and PTC developed powerful PTC’ activity within 10
0.05 M Fraction | 0.3 M Fraction
--- | ---
Normal plasma | 0.3 | 1.5 | 0.6 | 13
PTA' reagent | 6.0 | – | 0.4 | 0
BaSO₄ eluate | 0 | 0 | 30 | 31

Table 7.—The PTC' Activity of a 0.3 M Column Fraction of a BaSO₄ Eluate Measured in an Assay Containing Thrombin

<table>
<thead>
<tr>
<th>Test Reagent</th>
<th>PTC-intact with Thrombin (sec.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BaSO₄ eluate, 0.3 M fraction</td>
<td>33</td>
</tr>
<tr>
<td>Normal plasma, undiluted</td>
<td>113</td>
</tr>
<tr>
<td>Saline</td>
<td>&gt;300</td>
</tr>
</tbody>
</table>

Table 8.—The Effect of Calcium on PTC' Generation

<table>
<thead>
<tr>
<th>PTA' Source</th>
<th>PTC Source</th>
<th>Calcium</th>
<th>Incubation Time</th>
<th>Contact (sec.)</th>
<th>Intact (sec.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BaSO₄ eluate of PTC deficiency plasma</td>
<td>BaSO₄ eluate of HF deficiency plasma</td>
<td>–</td>
<td>0 days*</td>
<td>53</td>
<td>180</td>
</tr>
<tr>
<td>PTA' reagent</td>
<td>DEAE column</td>
<td>0 days</td>
<td>8 days</td>
<td>44</td>
<td>40</td>
</tr>
<tr>
<td>Saline control</td>
<td>DEAE column</td>
<td>–</td>
<td>0 days</td>
<td>65</td>
<td>300</td>
</tr>
<tr>
<td>PTA' reagent</td>
<td>DEAE column</td>
<td>–</td>
<td>0 days</td>
<td>66</td>
<td>328</td>
</tr>
</tbody>
</table>

*Reagents were aged separately for 8 days, then combined and assayed.
†PTC' generation assay technic was used for these 4 tests.

minutes in the presence of calcium but not in its absence. Therefore, calcium was added to all further reaction mixtures (see description of PTC' generation assay).

2. Evidence that the reaction between PTA' and PTC is independent of other clotting factors. The only detectable clotting contaminants in our PTA' preparations were traces of proconvertin and Stuart factor of the order of 0.1–0.2 per cent of a normal plasma reference standard. However, our PTC reagent prepared from normal plasma was contaminated with relatively large amounts of prothrombin, Stuart factor, and proconvertin (see table 9). In addition, some preparations contained traces of AHG and proaccelerin.

To determine whether or not these contaminating factors played a role in the generation of PTC' activity, special PTC reagents were prepared in each of which one of the contaminating factors was markedly reduced or absent. PTC reagents containing only 0.10–0.15 per cent of either Stuart factor or proconvertin activity were made by chromatographing the corresponding
Table 9.—Generation of PTC' Using PTC Reagents Deficient in Specific Clotting Factors

<table>
<thead>
<tr>
<th>PTC Source</th>
<th>Normal plasma</th>
<th>Proconvertin deficiency plasma</th>
<th>Stuart factor deficiency plasma</th>
<th>Artificial proaccelerin deficiency plasma</th>
<th>Exhausted serum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clotting factor content of PTC reagent*</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PTC-contact %</td>
<td>7</td>
<td>6</td>
<td>12</td>
<td>5</td>
<td>14</td>
</tr>
<tr>
<td>Prothrombin %</td>
<td>13</td>
<td>9.5</td>
<td>4.5</td>
<td>–</td>
<td>0.2</td>
</tr>
<tr>
<td>Stuart factor %</td>
<td>8</td>
<td>7.5</td>
<td>0.2</td>
<td>–</td>
<td>1.9</td>
</tr>
<tr>
<td>Proconvertin %</td>
<td>1.1</td>
<td>0.1</td>
<td>2.8</td>
<td>–</td>
<td>0.8</td>
</tr>
<tr>
<td>AHG %</td>
<td>–</td>
<td>–</td>
<td>1.5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Proaccelerin %</td>
<td>–</td>
<td>–</td>
<td>0.9</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

| Clotting time (sec.) PTC' generation assay |               |                                |                                |                                          |                 |
|-------------------------------------------|---------------|--------------------------------|                                |                                          |                 |
| 0 minutes incubation                      | 60            | 60                             | 63                             | 55                                       | 82              |
| 15 minutes incubation                     | 25            | 27                             | 26                             | 25                                       | 41              |

*Expressed as per cent of a normal plasma reference.
†Clotting times were converted to per cent PTC' activity by making a curve from dilutions of a maximally activated PTC' generation mixture containing PTC reagent from normal plasma. The activation ratio = % PTC' activity at 15 min./% PTC' activity at 0 min.

specific deficiency plasma. PTC fractions containing no detectable AHG or proaccelerin were made by chromatographing an artificial proaccelerin deficient plasma prepared by Stormorken’s technic. A PTC reagent containing only 0.2 per cent prothrombin was prepared by chromatographing artificial exhausted serum.

The PTC' activity obtained with these special PTC reagents is shown in table 9. No significant difference in PTC' generation was found, over a 15-minute period, using PTC reagents with markedly reduced to absent Stuart factor, proconvertin, proaccelerin, or AHG activities. Good PTC' activity also formed when the prothrombin-deficient PTC reagent was used but this preparation did not appear to activate quite as well as the others. The reason is unknown but is probably not its markedly reduced prothrombin content, since the degree of activation obtained with the other PTC reagents was clearly independent of the amount of contaminating prothrombin in each preparation. Thus, it would appear that under these conditions no other known clotting factor participates in the reaction between PTA' and PTC to form PTC'.

3. Evidence that PTC' is generated in a reaction in which PTA' acts as the enzyme and PTC as the substrate. To study the mechanism of the reaction, the generation of PTC' was measured in incubation mixtures containing varying concentrations of PTA' and PTC. The reagents used for these experiments had the following clotting activities:

(1) The PTA' reagent used for the experiments shown in figure 1 shortened the PTA' assay from a blank time of 135 seconds to 40 seconds. The reagent used for the experiments shown in figure 2 shortened the assay to 51 seconds.
The first reagent contained 2 per cent proaccelerin, 0.8 per cent proconvertin, and 0.1 per cent Stuart factor activity. The second reagent contained 0.2 per cent proaccelerin and proconvertin and 0.1 per cent Stuart factor. Neither reagent contained detectable amounts of other known clotting factors.

(2) The PTC reagent, which was made by chromatography of exhausted plasma to exclude any possibility of contamination with the precursors of PTA', contained 5 per cent PTC, 5.5 per cent prothrombin, 2 per cent Stuart factor and 0.8 per cent proconvertin activity. Its clotting time in the intact PTC assay exceeded 9 minutes.

The PTC' activity generated in the incubation mixture was measured in the PTC' generation assay, which included thrombin to eliminate artifact due to the generation of traces of thrombin in the incubation mixture. Clotting times were converted to per cent PTC' activity by reference to a curve made from dilutions of a maximally activated PTA'-PTC-calcium mixture in a saline-calcium solution. The clotting times of these dilutions plotted against concentration gave a straight line on log-log paper.

The effect upon PTC' generation of varying the concentration of PTA' while holding the concentration of PTC constant is shown in figure 1. It is clear that as the concentration of PTA' was decreased, the time required to generate maximum PTC' increased but the eventual amount of PTC' generated approached the same level. The converse experiment, varying the concentration of PTC while holding the concentration of PTA' constant, is shown in figure 2. Here one notes that as the concentration of PTC was reduced, the
total amount of PTC' generated decreased, but the time required to reach maximum PTC' activity remained the same. These data fit the hypothesis that PTA' is an enzyme and PTC a substrate.

To confirm this hypothesis, an attempt was made to isolate the products of the reaction. A fully activated mixture of PTA'-PTC-calcium was dialyzed overnight against starting buffer and then chromatographed on DEAE-cellulose. Fractions from the PTA region (0.05 M) and from the PTC region (0.30 M) were collected and examined in the PTA' assay and in the intact PTC assay with thrombin. As shown in table 10, PTA' activity was found in the PTA region—the expected result if PTA' were the unconsumed enzyme. The pooled fractions from this region yielded 9 per cent of the original PTA' added to the mixture. This is approximately the recovery we have obtained when PTA' reagent alone has been chromatographed. PTC'—an activity which shortens the intact PTC assay and which should also shorten the PTA' assay more than PTA' itself—was demonstrable in the fraction from the PTC region. Pooled tubes yielded 68 per cent of the original PTC' activity.

This PTC' fraction could not be further activated. A mixture of PTC' fraction-fresh PTA'-calcium examined immediately in the PTC' generation assay clotted in 75 seconds. After incubating for 15 minutes at 37 C., the mixture clotted in 85 seconds. Clearly, PTC capable of conversion to PTC' was not present in the incubation mixture. This is the expected result if PTC were the substrate, for all of the substrate should have been converted to the product, PTC', during the initial period of full activation prior to chromatography.

One thing more remained to be shown. Duckert has stated7 that one-stage
PTC assays based upon the PTT technic do not measure “genuine factor IX” activity—which he defines as activity correcting the defect of hemophilia B serum in the thromboplastin generation test—but measure, instead, an activity which he calls the prephase accelerator. Therefore, we added our PTC reagent and PTC’ generated from it to a thromboplastin generation test system containing hemophilia B serum. The results are shown in table 11. When a 1/10 dilution of normal serum was used as the test material, a minimum substrate clotting time of 9 seconds was obtained. When a mixture of PTA’-PTC-saline (a mixture incapable of generating PTC’ rapidly) was substituted for the normal serum, the minimum substrate clotting time was 10 seconds. When PTC’ was substituted, a minimum clotting time of 7.5 seconds was found. This PTC’ had been made by incubating the same PTA’ and PTC reagents with calcium for 20 minutes and then dialyzing the mixture overnight against saline to remove the calcium. It had high one-stage PTC activity (35 second clotting time in an intact PTC assay containing thrombin). It also appeared, as table 11 shows, to shorten the lag phase of the thromboplastin generation test. We conclude from these data that both our PTC reagent and the PTC’ generated from it had “genuine factor IX” activity and that the latter had, in addition, “prephase accelerator” activity.

**Discussion**

Initially, glass surfaces were thought to activate PTC directly. However, it is now clear that no such direct activation occurs. Glass surfaces activate Hageman factor, which then interacts with PTA to form the contact activation product. Ratnoff and his co-workers have presented evidence that the contact activation product is activated PTA (PTA’). Our chromatographic data support this conclusion, for native PTA and a PTA’ (activation product) reagent exhibited the same elution pattern from DEAE-cellulose. Recently, two groups of workers have presented good indirect evidence that PTA’ initiates the activation of PTC during intrinsic clotting in vitro.

The data reported herein provide direct evidence of the existence of an activated form of PTC (PTC’) which combines the coagulant effects of PTA’ and native PTC. PTC’ was demonstrated by chromatography of barium sulfate eluates of citrated plasma and by chromatography of a reaction mixture made up of PTA’ reagent, PTC reagent, and calcium. It is important to emphasize that the PTC’ isolated from this reaction mixture failed to activate further with fresh PTA’ and calcium.

Previous studies have emphasized that calcium is required for the activation
of PTC.\textsuperscript{3,5-9} We have found that PTC' can form slowly in calcium-free mixtures of PTA' and PTC. It appears that the function of calcium is to accelerate the reaction.

In agreement with Ratnoff and Davie\textsuperscript{9} our data suggest that PTA' and PTC are the only clotting factors required to form PTC'. From experiments using heparinized plasma, Egli and Schneider\textsuperscript{4} concluded that a small amount of clotting must take place before the HF-PTA reaction product can activate PTC. Ratnoff and Davie\textsuperscript{9} have shown that heparin interferes with the activation of PTC; this may account for Egli and Schneider's results. Nevertheless, it must be pointed out that whenever our solutions containing PTC' were tested, they were found to contain minute amounts of thrombin (i.e., of an activity which would eventually clot fibrinogen). The implications of the association of these two activities remain to be clarified.

The mechanism of activation of PTC was studied first by analyzing the kinetics of PTC' synthesis and then by isolating the reaction products. These experiments were based upon the assumption that since PTA' and PTC are both proteins, one probably served as an enzyme and the other as its substrate.

Formation of PTC' was studied in reaction mixtures containing varying concentrations of PTA' reagent and PTC reagent. When the concentration of the enzyme is reduced, the product, PTC', should form more slowly but eventually reach the same concentration as with higher concentrations of enzyme. In contrast, when the concentration of the substrate is reduced, the rate of synthesis should be affected little, but the amount of PTC' generated should fall. By these criteria, our data (figs. 1 and 2) fit the kinetic model, first proposed by Ratnoff and Davie,\textsuperscript{9} that PTA' is the enzyme catalyzing the conversion of PTC to PTC'. It is significant that our kinetic determinations are comparable to those of Ratnoff and Davie even though our reactants were prepared much differently. Our PTA' was made from plasma; theirs came from serum. Our PTC was prepared by column chromatography; theirs by BaSO\textsubscript{4} adsorption of oxalated plasma.

According to theory, enzymes are regenerated when product is formed. Therefore, after completion of the reaction one should be able to isolate enzyme and product, in this case PTA' and PTC'. When a maximally activated mixture of PTA', PTC, and calcium was chromatographed on DEAE-cellulose.

### Table 1.
The correction of the abnormal thromboplastin generation test of hemophilia B serum by PTC and PTC'.

<table>
<thead>
<tr>
<th>Test Material</th>
<th>Incubation Time (min.)</th>
<th>Clotting Time (sec.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>Saline</td>
<td>75</td>
<td>63</td>
</tr>
<tr>
<td>Normal serum 1/10</td>
<td>57</td>
<td>16</td>
</tr>
<tr>
<td>PTA' + PTC + saline*</td>
<td>53</td>
<td>20</td>
</tr>
<tr>
<td>PTC'\†</td>
<td>33</td>
<td>7.5</td>
</tr>
</tbody>
</table>

\*A mixture of PTA' reagent, PTC reagent, and saline in the ratio 1:1:0.1.

\†PTA' reagent, PTC reagent, and 0.315 M CaCl\textsubscript{2} were mixed in the ratio of 1:1:0.1. This mixture at zero incubation time clotted in an intact PTC assay with thrombin in 100 seconds. After 20 minutes incubation, it clotted in 36 seconds. The mixture was then dialyzed overnight against saline to remove calcium and used as the source of PTC'.
lose, two active areas were isolated. One contained PTA' activity; the other contained PTC' activity. Native PTC, capable of further activation, was not recovered. These data support the model, suggested by the kinetic data, that PTA' is the catalyst and PTC the substrate.

The relation between what we call PTC and PTC' and what Fisch and Duckert723 call “genuine factor IX” and prephase accelerator (PPA) requires comment. PTC and “genuine factor IX” both correct the defective thromboplastin generation test of PTC deficiency serum. In addition, our PTC preparations exhibit good activity when tested in one-stage assays providing maximum contact activation of the substrate. However, the Swiss workers state that “genuine factor IX” lacks one-stage PTC activity. It should be noted that they use an assay technic which provides only minimal contact activation (30 seconds exposure to the clotting tube surface). Moreover, instead of plasma, their standard reference is serum 2 hours old which, in our assays, has several times as much apparent PTC activity as plasma, probably because of its content of PTC'. With these conditions, i.e., clotting with minimal generation of PTA' and the use of a reference standard contaminated with PTC', it is not surprising that native PTC might appear to be defective in one-stage activity.

PTC' and PPA have much in common. Both require the contact activation reaction for their synthesis and both fail to form in plasma from a patient with hemophilia B. Both have high one-stage PTC activity and both also shorten the lag phase of the thromboplastin generation test. The major difference is that PTC' completely corrects the defective thromboplastin generation test of PTC deficiency serum, whereas the Swiss workers reported that PPA does not. Again, it seems likely to us that the difference may be quantitative rather than qualitative and related to the technic of the one-stage assay used to evaluate the strength of the PPA preparations added to their thromboplastin generation test system.23 It appears to us that PTC' and PPA will prove to be identical.

The discovery that PTC' may form in the absence of calcium (as in the BaSO₄ eluates of ciliated plasma) calls attention to the possibility that PTC' may contaminate PTC concentrates prepared for the treatment of hemophilia B. This would have several disadvantages. With some assay technics, it would lead one to believe that a concentrate contained much more PTC than it really possessed. Moreover, PTA' has been shown to be thrombogenic.15 Since PTC' incorporates in vitro PTA' activity, it is highly probable that PTC' is also thrombogenic. Indeed, we now suspect that PTC' is primarily responsible for the “serum thrombotic accelerator” activity of serum prepared as described by Reimer, Wessler, and Deykin.25 Finally, since PTC' is an active intermediate, one would think that it would not circulate but would be quickly removed by hepatic or reticuloendothelial clearance. For these reasons every effort should be made to prevent PTC' generation in preparing PTC concentrates and all concentrates should be tested for its presence.

**Summary**

1. An activated form of PTC (PTC') which incorporates the properties of both native PTC and activated PTA (PTA') has been identified in BaSO₄ eluates of intact citrated plasma.
2. PTC' has been synthesized from partially purified reagents in a reaction shown to require only PTA' and native PTC. Calcium ions greatly accelerate this reaction, but PTC' synthesis occurs slowly in their absence.

3. Kinetic and chromatographic analyses have shown that PTA' functions as an enzyme catalyzing the conversion of native PTC to PTC'.

4. On DEAE-cellulose chromatography, PTC and PTC' were found to elute at the same salt concentration.

**SUMMARIO IN INTERLINGUA**

PTC = componente thromboplastinic de plasma; PTC' = activate PTC; 
PTA = antecedente de thromboplastina de plasma; PTA' = activate PTA.

1. Un forma de PTC', con le proprietates de PTC native e de PTA', esseva identificate in eluatos a BaSO4 ex citratate plasma intacte.

2. PTC' esseva synthetisate ab partialmente purificate reagentes. Esseva trovate que le reaction require solmente PTA' e PTC native. Illo es grande-mente accelerate per iones de calcium, sed in le absentia de tal iones le synthese de PTC' progrede, ben qu lentemente.

3. Analyses cinetic e chromatographic ha monstrate que PTA' functiona como un enzyma-catalysta in le conversion de PTC native in PTC'.

4. In chromatographia a cellulosa-DEAE, il esseva trovate que le elution de PTC e de PTC' occurre al mesme concentration salin.

**REFERENCES**


IDENTIFICATION AND SYNTHESIS OF PTC


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The Identification and Synthesis of Activated Plasma Thromboplastin Component (PTC')

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