The Activation of Christmas Factor (Factor IX) by Activated Plasma Thromboplastin Antecedent (Activated Factor XI)*

OSCAR D. RATNOFF† AND EARL W. DAVIE

From the Departments of Medicine and Biochemistry, Western Reserve University, and University Hospitals of Cleveland, Cleveland, Ohio

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A study was performed on the factors influencing the activation of Christmas factor (plasma thromboplastin component or Factor IX) in human plasma. Evidence is presented indicating that the activation of Christmas factor occurs when it is incubated with activated plasma thromboplastin antecedent (activated PTA or activated Factor XI) and calcium. The effect of activated PTA appears to be enzymatic, and can be abolished by pretreatment of the activated PTA with diisopropyl phosphofluoridate. The activation of Christmas factor is also inhibited by heparin. The activated Christmas factor is relatively stable, and can be adsorbed onto aluminum hydroxide gel. The experiments described suggest that the activation of Christmas factor precedes the participation of antihemophilic factor (Factor VIII) in the clotting process.

Normal human plasma, depleted of its cells, clots readily in glass. The first recognized event leading to the coagulation of plasma is the activation of Hageman factor (Factor XII) by glass or certain other adsorbents (Ratnoff and Rosenblum, 1958; Biggs *et al.*, 1958; Lewis *et al.*, 1958; Vroman, 1958; Johnston *et al.*, 1958; Soulier *et al.*, 1959; Waaler, 1959). In turn, activated Hageman factor activates a second clot-promoting agent, plasma thromboplastin antecedent (PTA or Factor XI), presumably by an enzymatic process (Margolis, 1958; Soulier *et al.*, 1958; Hardisty *et al.*, 1959; Waaler, 1959; Ratnoff, 1960; Ratnoff *et al.*, 1961a). Neither the activation of Hageman factor nor that of PTA requires the presence of calcium ions. The suggestion has been made that the next step in clotting may be the activation of Christmas factor (plasma thromboplastin component or Factor IX) by activated PTA (Soulier *et al.*, 1958; Bachman *et al.*, 1958; Egli and Buscha, 1959; Biggs and Bidwell, 1959; Soulier, 1960; Duckert, 1960; Ratnoff *et al.*, 1961b), but this has not been established. These reactions are shown in equations (1), (2), and (3).

$$\text{Hageman factor} \xrightarrow{\text{glass}} \text{activated Hageman factor} \quad (1)$$

$$\text{PTA} \xrightarrow{\text{activated Hageman factor}} \text{activated PTA} \quad (2)$$

$$\text{Christmas factor} \xrightarrow[Ca^{++}]{\text{activated PTA}} \text{activated Christmas factor} \quad (3)$$

The present study adds to the evidence that activated PTA changes Christmas factor from an inactive to an active state [equation (3)]. The activation process requires the presence of calcium ions and is inhibited by heparin or diisopropyl phosphofluoridate. Activation occurs in the absence of Stuart factor (Factor X), antihemophilic factor (Factor VIII), pro-SPCA (Factor VII), proaccelerin (Factor V), prothrombin, fibrinogen, or platelets. Kinetic studies suggest that activated PTA is an enzyme which converts its substrate, Christmas factor, to an activated form.

NOMENCLATURE

The nomenclature used to describe the clotting factors in human blood was summarized recently (Ratnoff *et al.*, 1961a). The initial stages of the coagulation of plasma *in vitro* are thought to depend upon the interactions of four factors, namely, *Hageman factor* (Factor XII), *plasma thromboplastin antecedent* (*PTA* or Factor XI), *Christmas factor* (plasma thromboplastin component, PTC, or Factor IX) and *antihemophilic factor* (AHF or Factor VIII). These substances are believed to be deficient in the plasma of patients with Hageman trait, PTA deficiency, Christmas disease (or PTC deficiency), and classic hemophilia respectively. Evidence exists that Hageman factor and PTA can be altered to form *activated Hageman factor* and *activated PTA*. These factors, in association with Christmas factor, antihemophilic factor, calcium ions, platelets, Stuart factor (Factor X), and proaccelerin (Factor V), lead to the formation of active plasma thromboplastin. Active plasma thromboplastin then converts prothrombin to thrombin. Thromboplastin may also be derived from tissues; *tissue thromboplastin* acts optimally only in the presence of the precursor of serum prothrombin conversion accelerator (pro-SPCA or Factor VII) and Stuart factor.

The experiments to be described tested the ability of plasma or its fractions to shorten the abnormally long clotting time of plasma deficient in Christmas factor. The corrective effect which

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† Career Investigator of the American Heart Association.

developed in plasma or its fractions upon treatment in the manner to be described will be referred to as *Christmas factor activity* and will be attributed to the appearance of *activated Christmas factor*. We do not wish to imply that the corrective effect is necessarily due to the action of a single substance.

Materials and Methods

All studies were performed with blood, plasma, or plasma fractions which had been collected or prepared in silicone-coated tubes. Direct contact with glass or similar clot-promoting substances was avoided.

Venous blood was drawn from the antecubital veins of normal subjects or from patients with various coagulative disorders. No. 18 gauge needles coated with Arquad 2-C (Monocote-E, Armour) and glass syringes coated with silicone (G. E. Dri-Film SC-87) were used.

Platelet-deficient citrated plasma was prepared from blood to which one-ninth volume of 0.13 M trisodium citrate had been added (Ratnoff *et al.*, 1961a). *Unless otherwise noted, this platelet-deficient citrated plasma, which had had no contact with glass, was used in all the experiments to be described.*

Aluminum hydroxide–adsorbed plasma, deficient in prothrombin, pro-SPCA, Stuart factor, and Christmas factor, was prepared by incubating 10 volumes of plasma with one volume of aluminum hydroxide gel[1] for 3 minutes at 37° in silicone-coated Lusteroid tubes. The mixture was then centrifuged for 5 minutes at 2700 × *g* and the supernatant was used immediately.

Fibrinogen-deficient plasma was obtained by precipitating this protein from normal plasma by heating it at 50° for 30 minutes; the precipitate was removed by centrifugation. Such plasma contained no measurable proaccelerin but still retained appreciable antihemophilic activity.

Prothrombin-poor serum was prepared in sili cone-coated tubes from normal blood by the method of Alexander (1955) and mixed with one-fourth volume of 0.13 M sodium citrate solution. It was used immediately. Such serum contained no detectable prothrombin.

Crude PTA and crude activated Hageman factor were prepared by previously described methods from citrated plasma deficient in Hageman factor and PTA respectively (Ratnoff *et al.*, 1961a).

Crude activated PTA was prepared by incubating 0.9 ml of crude PTA and 0.1 ml of crude activated Hageman factor in a silicone-coated tube at 37° for 30 minutes (Ratnoff *et al.*, 1961a). This material accelerated the clotting of equal volumes of normal citrated plasma and 0.025 M calcium chloride solution from 20 to 7 minutes.

[1] Provided through the courtesy of Cutter Laboratories, Berkeley, Calif. The concentration of aluminum hydroxide gel is expressed by the manufacturer as aluminum oxide and contained 0.6% aluminum oxide.

Partially purified activated PTA was prepared from normal human serum in the following way. Venous blood was allowed to clot in glass tubes and the serum was separated by centrifugation. Serum in 1-liter batches was mixed for 10 minutes at room temperature with powdered barium sulfate (Baker) in a concentration of 100 mg per ml to deplete it of pro-SPCA, Christmas factor, Stuart factor, and any remaining prothrombin. The serum was heated at 56° for 30 minutes, to inactivate any remaining traces of these factors and of proaccelerin and antihemophilic factor, and then acidified by the addition of an equal volume of 0.15 M sodium acetate buffer (*p*H 5.2). The *p*H was adjusted to 5.2 by the addition of 1 M acetic acid and the acidified serum was then passed through a 6.5 × 12 cm carboxymethylcellulose column at room temperature. Occasionally, positive pressure was necessary to force the serum through the column. The column was washed with a volume of 0.15 M sodium acetate buffer equal to one half that of the original serum, and eluted with one-fourth volume of equal parts of 0.067 M sodium phosphate buffer (*p*H 6.8) and 1 M sodium chloride. The eluate was fractionated by the addition of solid ammonium sulfate, and collection of the material precipitated between 35–50% saturation. The precipitate was dissolved in one-fiftieth volume of 0.067 M sodium phosphate buffer, dialyzed against 4 liters of the same buffer, and lyophilized. Approximately 400 mg of material, about two fifths of it protein, was prepared from each liter of serum. The product was purified about 50- to 60-fold compared with the original serum with an apparent yield of 15%. This fraction contained little or no activated Hageman factor. Partially purified activated PTA retained its activity after storage in the lyophilized form at −25° for 30 months.

Barium sulfate eluates of normal plasma or plasma deficient in Christmas factor, pro-SPCA, or Stuart factor were prepared in silicone-coated equipment in the following manner. Venous blood was mixed with one-ninth volume of 0.1 M sodium oxalate solution, and the plasma was separated by centrifugation. The oxalated plasma was treated with 100 mg of powdered barium sulfate (Baker) per ml, centrifuged, and the barium sulfate washed twice with barbital-saline buffer. The barium sulfate was then eluted twice, each time with one-fourth volume of 10% solution of trisodium citrate dihydrate. The eluates were combined and dialyzed overnight at 4° against 0.15 tris(hydroxymethyl)aminomethane buffer at *p*H 7.45 (Sigma 121, Sigma Chemical Company).

Rabbit brain *thromboplastin* (Difco) was prepared by suspending 37 mg of thromboplastin per ml of 0.15 M sodium chloride solution, warming the mixture at 45° before use in accordance with the manufacturer's directions. The thromboplastin was used without filtration.

Rabbit brain "*cephalin*," a crude chloroform-

soluble fraction of acetone-dried brain, was prepared by the method of Bell and Alton (1954). This "cephalin" suspension was diluted 100-fold in barbital-saline buffer before use.

Carboxymethylcellulose, containing approximately 0.72 meq of acidic groups per gram of cellulose, was prepared by the procedure of Ellis and Simpson (1956).

Sodium *heparin* ("Liquemin," Organon), 10 mg (1000 USP units) per ml, was diluted to 1μg (0.1 unit) per ml with 0.15 M Tris buffer, *p*H 7.45.

Protein was determined by the method of Lowry *et al.* (1951). A fresh solution of crystalline bovine serum albumin was used as a standard.

Soy bean trypsin inhibitor (five times crystallized, Nutritional Biochemicals) was dissolved in 0.15 M Tris buffer, *p*H 7.45, at a concentration of 2 mg per ml.

Diisopropyl phosphofluoridate (DFP)–treated activated PTA was prepared by incubation of the partially purified activated PTA in barbital-saline buffer with various concentrations of DFP (K. and K. Labs., Long Island City, New York) at 25° followed by exhaust ve dia ysis against several changes of buffer at 4° or by a 10-fold dilution with buffer at 0°. Control samples without DFP were also incubated and subsequently treated in an identical manner.

Barbital-saline buffer was prepared as in earlier studies (Ratnoff *et al.*, 1961a). *Barbital-saline-citrate buffer* consisted of a mixture of 85% barbital-saline buffer and 15% 0.13 M trisodium citrate solution.

The *activation of Christmas factor* was tested by a two-step method. In the first step or *preliminary incubation*, the plasma or fraction to be tested was incubated with 0.017 M calcium chloride at 37°. After an appropriate interval, the calcium ions were bound by the addition of barbital-saline-citrate buffer either to the mixture itself or to an aliquot of the mixture. Depending upon the purposes of the experiment, the first step was performed in Pyrex or silicone-coated tubes. In the second step an *assay for activated Christmas factor* was performed by testing the effect of the citrated mixture upon the partial thromboplastin time of Christmas factor–deficient plasma (Langdell *et al.*, 1953). One-tenth ml of Christmas factor–deficient plasma was mixed in a silicone-coated tube with 0.1 ml each of the solution to be tested, "cephalin," and 0.05 M calcium chloride solution. The contents of the tube were mixed and the clotting time of the mixture was measured at 37°, the tubes being tilted continually. It should be emphasized that the concentration of the reactants during the preliminary incubation (step I) was much greater than in the assay for activated Christmas factor (step II). This difference in concentration helped to minimize additional activation of Christmas factor during the final assay.

In many experiments, a *measure of the degree of activation of Christmas factor* was obtained by preparing a standard curve in which the sample with the greatest activity was diluted serially with barbital-saline-citrate buffer. Each dilution was tested for activated Christmas factor and a curve drawn relating the concentration of the sample and the clotting times (partial thromboplastin times) induced by samples in Christmas factor–deficient plasma. An inverse linear relationship between the logarithms of the relative activities of the samples and the clotting times was obtained. A typical calibration curve is shown in Figure 1.

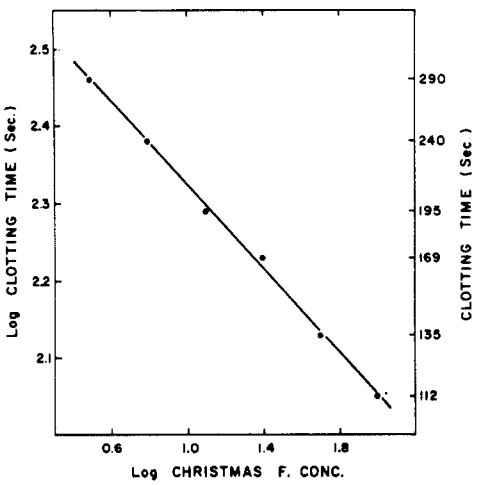

FIG. 1.—The effect of increasing concentrations of activated Christmas factor on the partial thromboplastin time of Christmas factor–deficient plasma. The logarithm of the clotting time in seconds is plotted against the logarithm of the activated Christmas factor concentration. The concentration of the sample with the greatest activity, *i.e.*, maximal activated Christmas factor, was arbitrarily set at 100.

Assays for the evolution of antihemophilic factor activity were performed by the same method used to test for activated Christmas factor, using a substrate of plasma deficient in antihemophilic factor.

The *evolution of thrombin* was measured by incubating a mixture of 0.1 ml of the aliquot to be tested and 0.2 ml of bovine fibrogen solution (Warner-Chilcott), said to contain 300 mg of coagulable protein per 100 ml, in Pyrex tubes at 37°.

All studies were performed in test tubes with an internal diameter of 8 mm unless otherwise noted.

RESULTS

I. *The Activation of Christmas Factor in Various Deficient Human Plasmas.*—In order to observe the activation of Christmas factor in a two-step experiment, various plasmas, each deficient in a specific clotting factor, were employed. This procedure permitted a study of the early stage of clotting, prior to the formation of thrombin, since in most cases the clotting time of the deficient plasmas was abnormally long.

When platelet-poor hemophilic plasma was

TABLE I

ACTIVATION OF CHRISTMAS FACTOR IN VARIOUS DEFICIENT PLASMAS

Preliminary Incubation Mixture[a]		Preliminary Incubation Period	Clotting Time[a] (sec.)
A.	Antihemophilic factor–deficient plasma	15 sec.	220
		16 min.	115
B.	Christmas factor–deficient plasma	15 sec.	359
		16 min.	353
C.	Hageman factor–deficient plasma	15 sec.	223
		20 min.	233
D.	PTA-deficient plasma	15 sec.	251
		20 min.	242

[a] 1.0 ml of plasma + 0.5 ml 0.05 M calcium chloride solution incubated in Pyrex tubes (internal diameter 11 mm) at 37°. At intervals, 0.1-ml aliquots were removed and mixed with 0.4 ml barbital-saline-citrate buffer. Each aliquot was then tested for Christmas factor activity by measuring its effect upon the recalcified clotting time (last column) of Christmas factor–deficient plasma in the presence of "cephalin."

incubated with calcium in Pyrex tubes, the concentration of *activated* Christmas factor increased with time (Table I). Similar results were observed in plasmas deficient in pro-SPCA, Stuart factor, proaccelerin, or fibrinogen and in prothrombin-deficient serum.

In contrast, activation of Christmas factor did not occur in plasma obtained from a patient with Christmas factor deficiency, nor in normal plasma treated with aluminum hydroxide gel, a procedure which depletes plasma of pro-SPCA, Christmas factor, Stuart factor, and prothrombin. Moreover, the activation of Christmas factor did not occur in plasmas deficient either in PTA or Hageman factor (Table I).

The activation of Christmas factor gradually increased with time, was much more rapid at 37° than at 0°, and did not occur unless calcium ions were present (Table II). Activated Christmas factor was formed in glass tubes, while virtually no activity evolved within 16 minutes in silicone-coated tubes.

The series of experiments demonstrated that the activation of Christmas factor occurred when plasma was recalcified and incubated in glass tubes. This activation required the presence of Christmas factor, PTA, and Hageman factor, but took place in the absence of platelets, added phospholipids, proaccelerin, pro-SPCA, and Stuart factor. Suitable control experiments demonstrated that the property which we have designated activated Christmas factor could not be attributed to the evolution of thrombin in the preliminary incubation mixture.

II. *The Effect of PTA and Hageman Factor on the Activation of Christmas Factor.*—In the preceding experiments the activation of Christmas factor occurred only when plasma was incubated in glass tubes. Glass accelerates clotting at least partially by "activating" Hageman factor, which then

TABLE II

EFFECT OF CALCIUM IONS ON THE ACTIVATION OF CHRISTMAS FACTOR IN HEMOPHILIC PLASMA

Preliminary Incubation Mixture[a]		Preliminary Incubation Period (min.)	Clotting Time[a] (sec.)
A.	Plasma + Ca^{++}	0	260
B.	Plasma + Ca^{++}	16	182
C.	Plasma − Ca^{++}	16	272

[a] 0.2 ml hemophilic plasma was incubated in Pyrex tubes at 37°. 0.1 ml of 0.025 M calcium chloride solution was added to tubes A and B at 0 time and to tube C after 16 minutes. The reaction was stopped by the addition of 1.2 ml of barbital-saline-citrate buffer to C at 0 minutes, and to B and C after 16 minutes. Each aliquot was then tested for Christmas factor activity by measuring its effect upon the recalcified clotting time (last column) of Christmas factor-deficient plasma in the presence of "cephalin."

appears to activate PTA (Ratnoff *et al.*, 1961a). The following experiments were designed to test whether activated PTA or activated Hageman factor enhanced the activation of Christmas factor.

Crude PTA was incubated in silicone-coated tubes with Christmas factor–deficient plasma and calcium. Only negligible Christmas factor activation occurred during the succeeding 20 minutes (Table III). Similarly, no Christmas factor activity evolved when crude *activated* Hageman factor was incubated with PTA-deficient plasma and calcium.

When crude activated PTA, prepared from preparations of PTA and activated Hageman

TABLE III

EFFECT OF ACTIVATED HAGEMAN FACTOR, PTA, AND ACTIVATED PTA ON THE ACTIVATION OF CHRISTMAS FACTOR

Preliminary Incubation Mixture[a]	Preliminary Incubation Period	Clotting Time[a] (sec.)
PTA-deficient plasma + activated Hageman factor	15 sec.	202
	20 min.	240
PTA-deficient plasma + PTA	15 sec.	240
	20 min.	238
PTA-deficient plasma + activated PTA	15 sec.	232
	12 min.	133

[a] 1.0 ml of PTA-deficient plasma was mixed with 0.5 ml of crude activated Hageman factor or crude PTA or crude activated PTA and 0.5 ml 0.05 M calcium chloride solution, and incubated at 37° in silicone-coated tubes (internal diameter 11 mm). At intervals, 0.1-ml aliquots were removed and mixed with 0.4 ml barbital-saline-citrate buffer. Each aliquot was then tested for Christmas factor activity by measuriug its effect upon the recalcified clotting time (last column) of Christmas factor–deficient plasma in the presence of "cephalin."

factor, was incubated with PTA-deficient plasma and calcium in silicone-coated tubes, a striking activation of Christmas factor occurred (Table III). In this experiment, low levels of activated PTA were employed in order to avoid thrombin and fibrin formation during the preliminary incubation step. These experiments, then, suggest that activated PTA participates directly in the activation of Christmas factor.

III. *The Effect of Partially Purified Activated PTA Upon the Activation of Christmas Factor.*—Further evidence that activated PTA activates Christmas factor was obtained in several different ways. Christmas factor activation occurred rapidly in hemophilic plasma incubated in silicone-coated tubes with partially purified activated PTA and calcium ions (Table IV). In the same way, Christmas factor activation was observed when activated PTA was incubated with plasma deficient in PTA or pro-SPCA.

TABLE IV

EFFECT OF PARTIALLY PURIFIED ACTIVATED PTA ON THE ACTIVATION OF CHRISTMAS FACTOR IN HEMOPHILIC PLASMA

Preliminary Incubation Period[a] (min.)	Clotting Time[a] (sec.)
0	161
4	118
8	112
12	105
16	88

[a] A series of silicone-coated tubes, each containing 0.1 ml of hemophilic plasma, 0.1 ml partially purified activated PTA, and 0.1 ml 0.025 M calcium chloride solution, were incubated at 37°. At intervals, 0.7 ml of barbital-saline-citrate buffer was added to successive tubes. Each mixture was then tested for Christmas factor activity by measuring its effect upon the recalcified clotting time (right-hand column) of plasma deficient in Christmas factor in the presence of "cephalin."

The effect of partially purified activated PTA was then tested upon crude preparations of Christmas factor. The barium sulfate eluate prepared from pro-SPCA-deficient plasma served as a source of Christmas factor; this eluate also contained Stuart factor, prothrombin, and other unidentified substances. The barium sulfate eluate of pro-SPCA-deficient plasma, otherwise untreated, contained little activated Christmas factor. When this eluate was incubated with activated PTA and calcium ions in silicone-coated tubes, the concentration of activated Christmas factor gradually increased. When the Christmas factor concentration was held constant and the concentration of activated PTA increased, only the *rate* of activation of Christmas factor increased, while the final concentration of activated Christmas factor approached the same final value

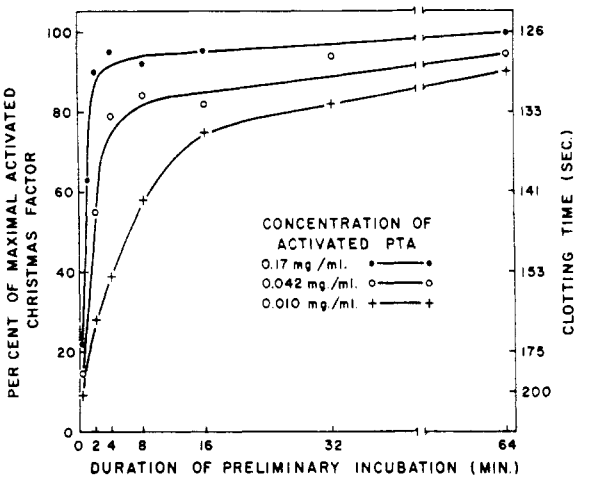

FIG. 2.—The influence of the concentration of activated PTA on the activation of Christmas factor in mixtures of activated PTA and crude Christmas factor. Three-tenths ml each of partially purified activated PTA (diluted serially with Tris buffer), barium sulfate eluate of pro-SPCA-deficient plasma (at one-half plasma concentration), and 0.025 M calcium chloride solution were incubated at 37° in silicone-coated tubes. At intervals, 0.1-ml aliquots were mixed with 0.4 ml of barbital-saline-citrate buffer and assayed for activated Christmas factor.

(Fig. 2). In the converse experiment, the concentration of Christmas factor was varied, while the concentration of activated PTA was kept constant. The reactions were allowed to proceed until the formation of activated Christmas factor was maximal. Under these conditions, the concentration of the activated Christmas factor was a function of the concentration of Christmas factor initially added (Fig. 3).

The same results were obtained when eluates were prepared from normal plasma or from plasma deficient in Stuart factor. Plasma deficient in prothrombin alone was not available. No activity evolved when an eluate prepared from Christmas factor–deficient plasma was incubated with activated PTA and calcium ions.

IV. *Inhibition of Activated PTA by DFP.*—In an earlier study, crude activated PTA was found to be inhibited by treatment with diisopropyl phosphofluoridate (Ratnoff *et al.*, 1961a). The effect of increasing concentration of DFP on partially purified activated PTA is shown in Figure 4. Preincubation with 3×10^{-5} M DFP for 20 minutes at 25°, *p*H 7.45, resulted in 50% loss of activity when Christmas factor–deficient plasma was employed for the assay.

Similar results were observed when the partially purified activated PTA treated with 10^{-4} M DFP was incubated in silicone-coated tubes with hemophilic plasma and Ca^{++}. Under these conditions, virtually no activation of Christmas factor occurred in the mixture. Thus, the inhibition in the two-step assay was essentially identical to that observed when the Christmas factor–

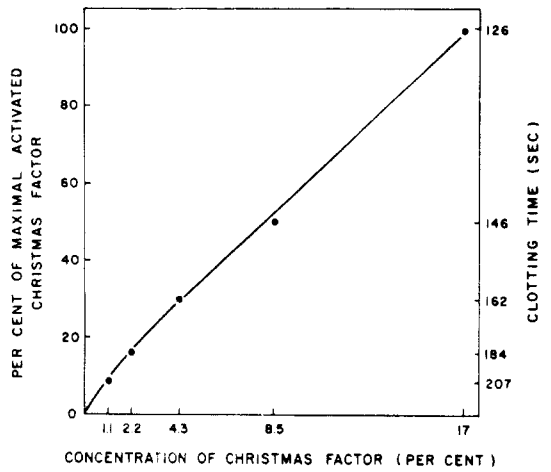

FIG. 3.—The influence of the concentration of Christmas factor on the activation of Christmas factor in mixtures of activated PTA and crude Christmas factor. Three-tenths ml each of partially purified activated PTA (0.5 mg per ml of Tris buffer), barium sulfate eluate of pro-SPCA-deficient plasma (diluted serially with Tris buffer) and 0.025 M calcium chloride solution were incubated at 37° in silicone-coated tubes for 64 minutes. At the end of this interval, 0.1-ml aliquots were added to 0.4 ml of barbital-saline-citrate buffer and assayed for activated Christmas factor activity. The concentration of Christmas factor is expressed as a percentage of the total amount added to the preliminary incubation mixture.

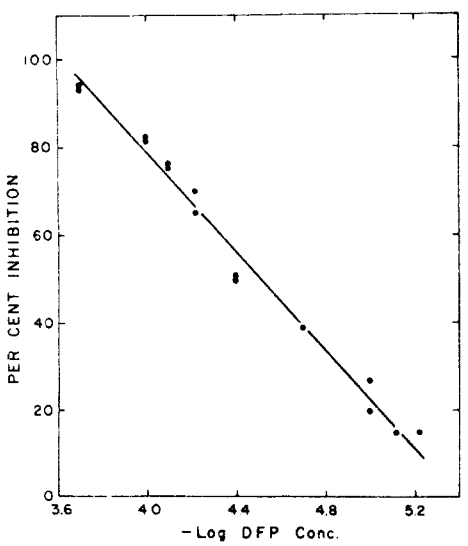

FIG. 4.—The inhibition of activated PTA by increasing concentrations of DFP. Each reaction mixture contained activated PTA, barbital buffer, and various concentrations of DFP; incubation for 20 minutes at 25° was followed by a ten-fold dilution in ice-cold buffer to terminate the reaction. Aliquots were assayed immediately with Christmas factor–deficient plasma and the inhibition determined by comparison with a calibration curve.

deficient plasma was used as an assay.

V. *The Effect of Heparin and of Soy Bean Trypsin Inhibitor Upon the Activation of Christmas Factor.*—Heparin, in a concentration of 0.025 units per ml, significantly inhibited the activation of Christmas factor in hemophilic plasma. This concentration of heparin in the preliminary incubation mixture was such that it did not interfere with the final assay of activated Christmas factor. Inhibition was also noted when heparin was added to a mixture of activated PTA and Christmas factor prepared from the barium sulfate eluate of pro-SPCA-deficient plasma (Table V).

TABLE V

EFFECT OF HEPARIN ON THE ACTIVATION OF CHRISTMAS FACTOR

Preliminary Incubation Mixture[a]	Preliminary Incubation Period (min.)	Clotting Time[a] (sec.)
Christmas factor + activated PTA + heparin	0 16	229 235
Christmas factor + activated PTA + buffer	0 16	229 158

[a] 0.1 ml of barium sulfate eluate of pro-SPCA-deficient plasma (at one-half original plasma concentration), 0.1 ml of activated PTA (0.03 mg/ml), 0.1 ml heparin (0.1 unit per ml of Tris buffer) *or* 0.1 ml Tris buffer, and 0.1 ml 0.025 M calcium chloride solution were incubated in silicone-coated tubes at 37°. At the end of the incubation period, 1.0 ml barbital-saline-citrate buffer was added to each tube, and 0.1 ml of buffer to the tube containing heparin and *vice versa*. Each mixture was then tested for Christmas factor activity by measuring its effect upon the recalcified clotting time (last column) of Christmas factor–deficient plasma in the presence of "cephalin."

Unlike heparin, soy bean trypsin inhibitor, in a concentration of 0.5 mg per ml, was without effect upon the evolution of Christmas factor activity in hemophilic plasma.

VI. *The Activation of Antihemophilic Factor in Christmas Factor–Deficient Plasma and Stuart Factor–Deficient Plasma.*—One current hypothesis (Biggs and Bidwell, 1959) suggests that the initial steps in clotting require the successive participation of Hageman factor, PTA, Christmas factor, and antihemophilic factor. In Section I, data were presented that the activation of Christmas factor occurred in the absence of antihemophilic factor or Stuart factor. The following experiments were designed to test the converse possibility, namely, whether the development antihemophilic factor activity requires the presence of Christmas factor.

Plasma deficient in Christmas factor was recalcified in Pyrex tubes and incubated at 37°. Successive samples of this mixture were assayed for antihemophilic factor. In repeated experi-

ments no increase in antihemophilic factor activity was detectable within a period of 18 minutes (Table VI). In contrast, when Stuart factor-deficient plasma was treated in an identical manner, a marked activation of antihemophilic factor was observed after an 8-minute preliminary incubation (Table VI).

TABLE VI

THE ACTIVATION OF ANTIHEMOPHILIC FACTOR IN CHRISTMAS FACTOR-DEFICIENT PLASMA AND STUART FACTOR-DEFICIENT PLASMA

Preliminary Incubation Mixture[a]	Preliminary Incubation Period (min.)	Clotting Time[a] (sec.)
Christmas factor-deficient plasma	0	197
	18	195
Stuart factor-deficient plasma	0	342
	8	147

[a] 2.0 ml of Christmas factor-deficient plasma or Stuart factor-deficient plasma and 1.0 ml of 0.05 M calcium chloride solution were incubated in a Pyrex tube (internal diameter 11 mm) at 37°. At intervals, 0.4-ml aliquots were withdrawn and mixed with 1.6 ml barbital-saline-citrate buffer in an iced silicone-coated tube and assayed for antihemophilic factor activity.

VII. *Some Properties of "Activated" Christmas Factor.*—The activated Christmas factor which was formed in hemophilic plasma was relatively stable. Hemophilic plasma was recalcified and incubated for 16 minutes at 37° in Pyrex tubes in the manner described. The mixture was then diluted with four volumes of barbital-saline-citrate buffer. No loss of activated Christmas factor was detectable when this diluted mixture was incubated at 37° for 200 minutes. At 56°, about 90% of its activity was lost within 5 minutes, and 99% within 30 minutes. All measurable activity disappeared when the diluted mixture was treated with one-tenth volume of aluminum hydroxide gel. In contrast to activated PTA, activated Christmas factor is not inhibited by DFP.

The increase in Christmas factor activity which occurred in recalcified plasma or in barium sulfate eluates treated with activated PTA indicate that these procedures alter the Christmas factor molecule. Thus far it has not been possible to separate this activated Christmas factor from a precursor form by means of column chromatography with a number of ion-exchange cellulose resins. This suggests that only a very small modification of Christmas factor occurs during its activation by activated PTA.

DISCUSSION

The sequence of changes leading to the coagulation of platelet-free human plasma is gradually becoming clearer. The first step which has been determined is the activation of Hageman factor (Factor XII) upon contact with certain adsorbents (Ratnoff and Rosenblum, 1958; Biggs *et al.*, 1958; Lewis *et al.*, 1958; Vroman, 1958; Johnston *et al.*, 1958; Soulier *et al.*, 1959; Waaler, 1959). Next, the activated Hageman factor seems to convert PTA (Factor XI) from an inactive to an active form (Margolis, 1958; Soulier, *et al.*, 1958; Waaler, 1959; Hardisty and Margolis, 1959; Ratnoff, 1960; Ratnoff *et al.*, 1961a).

Several lines of evidence support the view that the next step in clotting is the activation of Christmas factor (Factor IX) by activated PTA (Bachman *et al.*, 1958; Egli and Buscha, 1959; Biggs and Bidwell, 1959). Bachmann *et al.* (1958) described two patients with PTA deficiency whose plasma seemed partially deficient in Chritmas factor. This abnormality was corrected by the addition of Christmas factor-deficient serum. Presumably the Christmas factor-deficient serum provided something lacking in PTA-deficient plasma and needed for the detection of Christmas factor.

The converse experiment was performed by Biggs and Bidwell (1959), who drew similar conclusions. They observed that the clotting defect of serum deficient in Christmas factor was only imperfectly corrected by the serum of patients with PTA deficiency. If, however, the two serums were incubated together for an hour before testing, the clotting defect was completely corrected. Biggs and Bidwell deduced that the initial stages of clotting result in the production of active Christmas factor from a precursor.

Experiments of Egli and Buscha (1959) were interpreted the same way. They treated human serum with barium sulfate, a process which removes pro-SPCA, Christmas factor, Stuart factor, and any residual prothrombin. This adsorbed serum, treated with glass to activate Hageman factor and PTA, was mixed with untreated serum. Egli and Buscha concluded that Hageman factor and PTA activated by contact with glass, reacted with Christmas factor, a process requiring the presence of calcium ions.

The present study furnishes additional evidence that activated PTA activates Christmas factor. Unlike previous studies, this study used partially purified reagents to follow the evolution of Christmas factor activity, which was tested upon a substrate of plasma deficient in this substance. Platelet-poor human plasma acquired Christmas factor activity when incubated with calcium ions in Pyrex tubes. This activity appeared in plasmas deficient in each of the recognized clotting factors except Hageman factor, PTA, and Christmas factor. No such evolution of activity occurred when normal plasma was incubated in silicone-coated tubes. These observations suggested that the activation of Hageman factor and PTA preceded the appearance of activated Christmas factor.

The role of PTA and Hageman factor was fur-

ther clarified by incubating PTA-deficient plasma with preparations of these substances. PTA-deficient plasma contains Hageman factor, but this is inactive if the plasma is prepared and tested in silicone-coated tubes. The addition of PTA did not induce Christmas factor activity in such PTA-deficient plasma. Nor did *activated* Hageman factor induce Christmas factor activity in PTA-deficient plasma, since the mixture lacked a source of PTA. When PTA-deficient plasma was incubated with *activated* PTA, however, Christmas factor activity quickly developed.

Christmas factor activity also developed when preparations of activated PTA were incubated with crude Christmas factor. We have been unable to prepare Christmas factor devoid of all other clotting activities; repeated efforts have resulted only in the simultaneous purification of two other vitamin K–dependent clotting factors (*i.e.*, pro-SPCA and Stuart factor). However, crude Christmas factor could be prepared readily by adsorbing oxalated plasma with barium sulfate and eluting the barium sulfate with sodium citrate solution. This Christmas factor prepared from pro-SPCA-deficient plasma was contaminated with prothrombin and Stuart factor as well as other unidentified factors. Activated Christmas factor was readily evolved by incubation with activated PTA. The same effect could be obtained by preparing barium sulfate eluates of normal plasma or plasma deficient in Stuart factor, but eluates of Christmas factor-deficient plasma were inactive. These observations indicate that the action of activated PTA upon barium sulfate eluates depends upon their content of Christmas factor, that is, upon the presence of a substance or property deficient in the plasma of patients with Christmas disease.

The mechanism through which activated PTA induces the development of Christmas factor activity was clarified in simple kinetic experiments. The activation of Christmas factor appeared to be much more rapid at 37° than 0°. The rate at which activation of Christmas factor occurred was a function of the concentration of activated PTA in the incubation mixture. The magnitude of the activity which could be evolved was proportional to the concentration of Christmas factor in the incubation mixture. These experiments are compatible with the hypothesis that activated PTA is an enzyme which catalyzes the activation of Christmas factor, possibly by limited proteolysis. The inhibition of activated PTA by diisopropyl phosphofluoridate is consistent with this view, and suggests that activated PTA may be an esterase. Preliminary experiments employing radioactive DFP have shown a direct binding of the inhibitor to the protein. The nature of the binding site of the diisopropyl phosphoryl group to the protein is now under investigation.

In all of the experiments described, the development of Christmas factor activity required the presence of calcium ions, a dependence previously suggested by Bergsagel (1955) and by Egli and Buscha (1959). In contrast, the activation of Hageman factor and PTA occurs in the absence of calcium ions (Ratnoff *et al.*, 1961a). Bergsagel (1955) assumed that the activation of Christmas factor involves the formation of a complex between this substance and calcium. Our data suggest that this is unlikely. Although the addition of citrate ions could halt the activation of Christmas factor, whatever activity had evolved until then was stable. It seems more likely that calcium ions are a necessary co-factor for the action of activated PTA upon the Christmas factor substrate.

O'Brien (1958, 1960), Shanberge *et al.* (1959), and Greig (1959) have described experiments interpreted to mean that heparin inhibits the action of Christmas factor in thromboplastin generation. We were unable to demonstrate that heparin inhibited Christmas factor once this substance had been activated. On the other hand, heparin strongly inhibited the activation of Christmas factor by activated PTA. How this inhibition comes about has not been studied. It seems unlikely that it is related to the binding of calcium ions by the sulfuric acid radicals of the heparin molecule, for the concentration of heparin which was effective was relatively small compared with the concentration of calcium in the reaction mixture.

Many of the experiments described in the present report were performed with hemophilic plasma. The advantage of using hemophilic plasma is due to the fact that such plasma clots only after a long delay. In this way, it was possible to isolate the early steps in the clotting process without obvious interference from the formation of thrombin. By using plasmas obtained from patients with various congenital coagulative abnormalities, it was possible to formulate a tentative concept of the order in which the various factors reacted. In experiments not included in the present report, it has been observed that the only clotting activities which evolved in recalcified Christmas factor–deficient plasma were small amounts of PTA and Hageman factor. Recalcified hemophilic plasma acquired, in addition, potent Christmas factor activity. Plasma deficient in Stuart factor developed, in addition, increasing amounts of antihemophilic activity. Such observations provide the basis for the tentative scheme portrayed in Figure 5. Since a deficiency of Hageman factor is not associated with bleeding difficulties, it is likely that coagulation may also be initiated through other mechanisms than those proposed in this scheme. It is clear from the nature of these experiments that the activities described need not be due to discrete clotting factors. They may well be due to the cumulative effect of various substances, active at different stages in

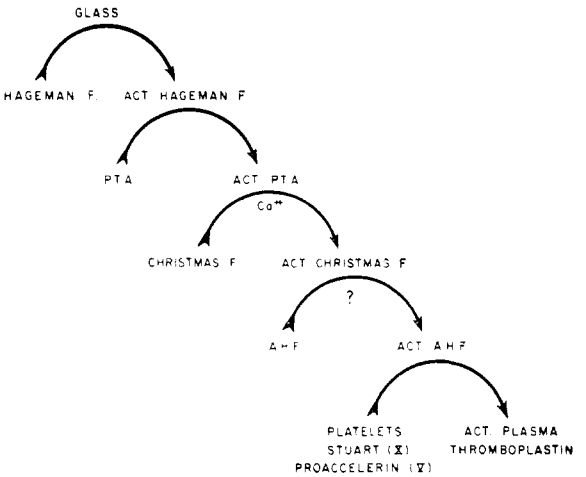

FIG. 5.—A possible mechanism for the successive steps leading to the formation of "active plasma thromboplastin" in human plasma.

the clotting process. Indeed the presence of activated clotting factors whose site of action is late in the clotting process seriously impairs our ability to test earlier steps in the chain. It should be emphasized that the studies reported in this paper were performed with human plasmas. That similar reactions occur in other mammalian plasmas can only be inferred from their similarity to human plasma. The mechanisms which operate to induce coagulation in avian plasma may well be significantly different, since such plasma appears to be deficient in Hageman factor (Ratnoff and Rosenblum, 1958), PTA (Wartelle, 1958), and Christmas factor (Didisheim *et al.*, 1959).

These studies reinforce the view, expressed in our previous papers, that only with the isolation and purification of the various clotting factors in their inactive states will it be possible to define blood clotting in chemical terms. What has begun to emerge is a series of enzymatic reactions ultimately leading, through steps still largely undefined, to the formation of the fibrin clot.

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