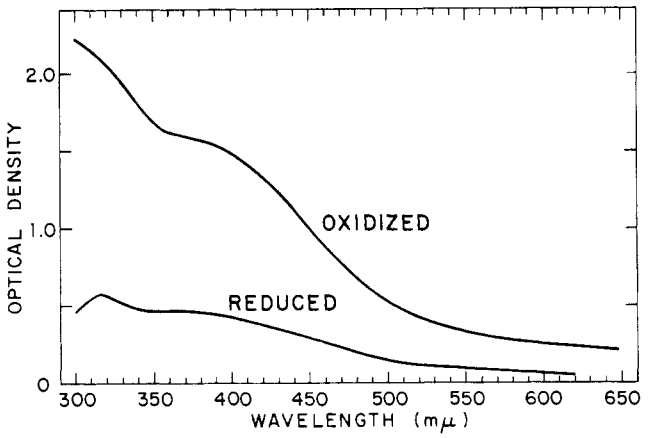

FIG. 2.—Absorption spectra of aqueous solution of oxidized and reduced ferredoxin, 0.027% concentration.

at room temperature for 4 hours. The concentration of the substrate is chosen so that the absorption spectra of the initial and final reduced products can be readily measured in a spectrophotometer (i.e., a 1-cm path length with an optical density of 1–2 at the absorption maximum).

With the exception of ferredoxin (obtained from Drs. E. Knight and D. Blomstrom, Central Research Department, Du Pont Co., Wilmington, Del.), the materials studied were all commercial-grade samples (Nutritional Biochemicals Corp., Cleveland, Ohio). The oxidized cytochrome *c* and methemoglobin were prepared from the reduced forms by oxidation with potassium ferricyanide and purified by rigorous dialysis. The formamidine sulfinic acid was obtained from Aldrich Chemical Co., Milwaukee, Wisc. In the case of methemoglobin, a steady stream of nitrogen was bubbled through the sample during the course of the reaction.

The results for ferredoxin are shown in Figure 2. Here, the reduction was carried out under nitrogen to prevent the rapid return to the starting material by oxidation with oxygen from the air. The course of the reduction was followed spectrophotometrically and was shown to come to equilibrium in about 4 hours. The reaction could be speeded up to 5 minutes by heating to 40° at *p*H 7. The reduction of peroxidase was also extremely sensitive to oxygen from the air; in fact, a small amount of peroxidase behaved as a good catalyst for converting formamidine sulfinic acid to the sulfonic acid form in the presence of air. In every case, the published literature spectral data were used to confirm the formation of the reduced product.

These experiments illustrate the application of formamidine sulfinic acid as a suitably mild reagent for converting certain oxidized biochemical substrates to their reduced forms; it is particularly useful where the reaction is to be studied by ultraviolet (above 300 mμ) or visible spectroscopy.

REFERENCES

Böeseken, J., (1936), *Rec. Trav. Chim. 55*, 1040.
Golunyad, V. A., and Bolovtova, C. T. (1962), *Chem. Abstr. 56*, 9696b.
Gore, P. H., (1954), *Chem. Ind. (London)*, 1355.
Shashoua, V. E., (1964), *Nature 204* (in press).

The Activation of Antihemophilic Factor (Factor VIII) by Activated Christmas Factor (Activated Factor IX)*

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The reaction of activated Christmas factor with antihemophilic factor has been investigated. Evidence is presented indicating that activated Christmas factor is an enzyme which reacts with antihemophilic factor converting the latter to an activated product which accelerates clotting. The reaction requires the presence of calcium ions and phospholipid. For the latter requirement, a mixture containing equal amounts of phosphatidylcholine and phosphatidylserine is most effective. Diisopropylphosphofluoridate and soybean-trypsin inhibitor have no effect on the activation of antihemophilic factor, whereas heparin is a potent inhibitor. Preincubation of activated Christmas factor with thorium tetranitrate also inhibits the reaction. Evidence for the participation of phospholipid in a reaction following the activation of antihemophilic factor is also provided.

The requirement of antihemophilic factor (AHF[1] or factor VIII) for normal blood clotting in the intrinsic system is well established. In hemophilic plasma the formation of thrombin is slow (Addis, 1911), and prothrombin consumption is greatly impaired (Brinkhous, 1939). Antihemophilic factor participates in the clotting sequence after the interaction of Hageman factor (factor XII), plasma thromboplastin antecedent (PTA or factor XI), and Christmas factor (PTC or factor IX) (Ratnoff and Davie, 1962). The initial reactions are shown in equations (1), (2), and (3).

$$\text{Hageman f.} \xrightarrow{\text{surface}} \text{activated Hageman f.} \quad (1)$$

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

$$\text{Christmas f.} \xrightarrow[Ca^{2+}]{\text{activated PTA}} \text{activated Christmas f.} \quad (3)$$

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[1] Abbreviations used in this work: AHF, antihemophilic factor; PTA, plasma thromboplastin antecedent; PTC, Christmas factor, plasma thromboplastin component; DFP, diisopropylphosphofluoridate.

Bergsagel and Hougie (1956) demonstrated that Christmas factor, AHF, Stuart factor (factor X), and calcium ions interact to form an activated product (product I), which in the presence of washed platelets would accelerate the clotting of recalcified AHF- and Christmas-deficient plasma. The activated product did not correct proaccelerin-(factor V) deficient plasma.

Reactions involving AHF have also been studied by Biggs and Bidwell (1959) and Fisch and Duckert (1959). These investigators have studied the kinetics of product I formation and its relationship to AHF.

Generally, it has been assumed that activated Christmas factor activates AHF and the latter, in turn, activates Stuart factor, yielding product I. Experimental proof for these reactions, however, has not yet been provided. In the present paper, the activation of AHF by activated Christmas factor has been studied under conditions which do not yield product I. Activated Christmas factor has been shown to be an enzyme which converts AHF to activated AHF in the presence of phospholipid and calcium ions.

Materials and Methods

Standard *ecteola cellulose* with a capacity of 0.3 meq of base/g was purchased from Carl Schleicher and Schuell Co., Keene, N. H. *G-25 Sephadex* (coarse grade) was purchased from Pharmacia Laboratories, Inc., Rochester, Minn. Crystalline *soybean-trypsin inhibitor* was purchased from Nutritional Biochemicals Corp., Cleveland, Ohio. *Diisopropylphosphofluoridate* (DFP) was purchased from K and K Laboratories, Long Island City, N. Y. Bovine *fibrinogen* was purchased from Warner-Chilcott, Morris Plains, N. J. Rabbit brain *thromboplastin* (Difco Laboratories, Detroit, Mich.) was prepared according to manufacturer's instructions by suspending the thromboplastin in 0.15 M NaCl (37 mg/ml) and warming the mixture at 45° for 10 minutes.

Human plasmas deficient in antihemophilic factor, Stuart factor, Christmas factor, and proaccelerin were kindly provided by Drs. C. Hougie, M. Gaston, and O. D. Ratnoff. Assays for antihemophilic factor, Stuart factor, Christmas factor, and proaccelerin were done by previously published methods (Breckenridge and Ratnoff, 1962; Ratnoff *et al.*, 1961).

Bovine AHF was purchased from S. Maw and Sons, Ltd., Barnet, England, and contained about 20% protein on a weight basis. The authors wish to thank Dr. Cecil Hougie for kindly providing this material for the early phases of this work. This product, when tested at a protein concentration of 1.1 mg/ml, was free of proaccelerin, Stuart factor, and thrombin, but contained substantial amounts of fibrinogen. Prior to use, 100 mg of crude AHF was dissolved in 1 ml of 0.025 M Tris–0.025 M imidazole buffer, *p*H 6.5. The solution was passed through a Sephadex column (1.5 × 22 cm) previously equilibrated with the same buffer at 4°. Eluate fractions were tested for protein by combining a 0.2-ml aliquot with 1 ml of 10% trichloroacetic acid. The first 6 ml of protein-containing eluate (2–3 mg protein/ml) was used immediately for the activation studies.

Activated PTA was prepared from human serum by the method of Ratnoff and Davie (1962). Crude *Christmas factor* was prepared from prothrombin-poor bovine serum. Blood was collected at the slaughterhouse within 3 minutes after death and immediately was mixed with 0.03 volume of Difco thromboplastin according to the method of Alexander (1955). The blood was allowed to clot for 48–60 hours at room temperature, after which the serum was collected. Only preparations free of thrombin and prothrombin at this point were utilized for further fractionation. $BaSO_4$ eluates containing potent Christmas-factor activity were prepared by mixing 100 mg of Baker $BaSO_4$ per ml of serum and stirring for 15 minutes at room temperature. The suspension was centrifuged and the $BaSO_4$ precipitate was washed with cold 0.15 M NaCl (one-fourth the original volume) and recentrifuged. After a second saline wash of the $BaSO_4$ precipitate, the Christmas factor was extracted from the $BaSO_4$ precipitate by stirring with a 10% (w/v) solution of trisodium citrate dihydrate (one-fifth the original volume) for 10 minutes at room temperature. The eluate collected after centrifugation was stored in aliquots at −20°. The following operations were carried out at 4°. Prior to use, a 12-ml aliquot containing approximately 60 mg of protein was thawed and dialyzed for 10–12 hours against several changes of 0.05 M Tris, *p*H 8.6. Then it was passed through an *ecteola cellulose* column (1.8 × 42 cm) previously equilibrated with the 0.05 M Tris buffer. The column was prepared by suspending 10 g of ecteola cellulose in 500 ml of 0.05 M Tris–3.0 M NaCl, *p*H 8.6. The column was then washed with 1–2 liters of 0.05 M Tris buffer, *p*H 8.6. Protein was eluted from the column with the wash buffer and fractions were collected in 10-ml aliquots. Protein concentrations were determined by absorption at 280 mμ and the tubes containing the first protein peak (near the solvent front) were combined and absorbed with $BaSO_4$ (final concentration of 100 mg/ml). The partially purified Christmas factor was eluted from $BaSO_4$ as previously described, dialyzed against 500 volumes of 0.15 M NaCl at 4° for 10 hours, and used immediately in the activation experiments. This preparation, while depleted of prothrombin, probably contains significant amounts of Stuart factor and factor VII.

For the thorium-inhibition experiments, Christmas factor was prepared by a modification of the procedure of White *et al.* (1953). Bovine prothrombin-poor serum was incubated at 37° for 2 hours. The *p*H of the serum was then lowered to 2.9 by the addition of 1 N HCl. This acidified preparation was incubated at 37° for 4 hours, after which the *p*H was raised to 7.2 with 1 N NaOH. Denatured protein was removed from the serum by centrifugation and the supernatant was absorbed with $BaSO_4$ (100 mg/ml of serum preparation). Christmas-factor activity was eluted from the $BaSO_4$ precipitate as described above. The eluent was concentrated by the addition of saturated $(NH_4)_2SO_4$ solution to a final concentration of 50% followed by centrifugation at 20,000 × *g* for 15 minutes. The $(NH_4)_2SO_4$ precipitate was dissolved in a minimal volume of 10% (w/v) solution of trisodium citrate dihydrate and stored in aliquots at −20°. The preparation was thawed and dialyzed for 10 hours against 500 volumes of 0.15 M NaCl at 4° prior to use. This preparation contained no detectable Stuart-factor activity.

Platelet-deficient citrated human plasma was prepared in silicone glassware from blood to which 0.10 volume of 0.13 M trisodium citrate had been added (Ratnoff *et al.*, 1961). This plasma, which had no contact with a glass surface, was stored at −20°.

Crude phospholipid ("Centrolex P") was purchased from Central Soya, Chicago, Ill. A 0.10% (w/v) suspension was prepared by homogenization of the crude phospholipid in 0.15 M NaCl and aliquots were stored in Lusteroid tubes at −20°. Phosphatidylserine and phosphatidylcholine were kindly provided by Drs. D. Papahadjopoulos and D. J. Hanahan as a suspension in a buffer containing 0.02 M Tris and 0.02 M maleate, *p*H 7.0, at a concentration of 1.7×10^{-3} M. The prep-

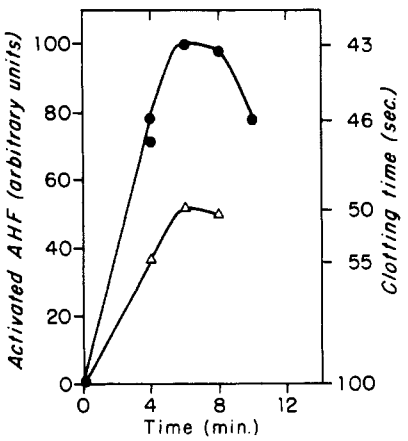

FIG. 1.—The formation of activated AHF with time at two different concentrations of AHF. Reactions were incubated as described under Table I. ●—● contained 1.8 mg AHF/ml and △—△ contained 0.9 mg AHF/ml.

aration and properties of these compounds have been described elsewhere (Papahadjopoulos *et al.*, 1962).

Sodium *heparin* (Invenex, San Francisco, Calif., 1000 USP units/ml) was diluted to 1.0 unit/ml with 0.15 M Tris buffer, *p*H 7.5. *Protein* was determined by the biuret procedure of Gornall *et al.* (1949), employing a freshly prepared solution of crystalline bovine serum albumin as the standard. The presence of thrombin was measured by incubating a 0.1-ml test sample with 0.2 ml of bovine fibrinogen solution containing 3.0 mg coagulable protein per ml at 37°. The clotting time was determined with continual tipping.

The activation of AHF was studied in a three-stage incubation. In the first incubation, activated PTA was incubated with Christmas factor and calcium ions for 15 minutes at 37°, permitting maximal Christmas-factor activation (Ratnoff and Davie, 1962). A typical reaction mixture contained 0.05 ml of activated PTA (1.4 mg/ml), 0.4 ml of Christmas factor (3.0 mg/ml), 0.25 ml of 0.15 M Tris buffer, *p*H 7.5, and 0.1 ml of 0.05 M $CaCl_2$. An aliquot of this reaction mixture diluted 1:10 in cold 0.15 M Tris buffer, *p*H 7.5, decreased the clotting time of normal recalcified platelet-deficient plasma in the presence of phospholipid in silicone tubes from 100 seconds (zero time) to 50 seconds (15 minutes).

In the second incubation at 24° crude activated Christmas factor was diluted 1:5 in 0.15 M Tris buffer, *p*H 7.5, prior to use. A 0.025-ml aliquot of the diluted activated Christmas factor (0.3 mg/ml) was added to a tube containing 0.4 ml of AHF (2.25 mg/ml), 0.05 ml of 0.05 M $CaCl_2$, and 0.025 ml of purified phospholipid (3.4×10^{-4} M).

In the third incubation, the activated product was examined by testing its acceleration of the partial-thromboplastin time of normal plasma in silicone tubes. Generally, a 0.05-ml sample of the second incubation mixture was diluted in Tris buffer (0.15 M, *p*H 7.0), 1:20 or 1:50, and then added to 0.1 ml test plasma, 0.1 ml phospholipid suspension ("Centrolex P"), and 0.1 ml of 0.025 M $CaCl_2$. The contents of the tube were rapidly mixed and the clotting time was determined at 37° with continual tipping. Tris-buffer controls always gave clotting times longer than 250 seconds. Each assay was run in duplicate.

The *degree of activation* was determined from a standard curve prepared immediately from the sample with the greatest activity. This sample was diluted to several concentrations in Tris buffer (0.15 M *p*H 7.5) and the clotting times for the diluted fractions were tested. A straight-line relationship was obtained when the logarithm of the clotting time was plotted against the logarithm of the concentration of activated product. In each series of experiments the fraction with maximal activity was arbitrarily expressed as 100 units of activated AHF.

RESULTS

Formation of an Activated Product from Antihemophilic Factor.—In order to study the activation of AHF, three separate reactions are required: The first involves the activation of Christmas factor (Ratnoff and Davie, 1962), the second, the activation of AHF, and the third, the assay of activated AHF with substrate plasma containing calcium ions and phospholipids. Experiments demonstrating the factors required for production of an activated product in the presence of AHF are shown in Table I. In the presence of phospholipid and calcium ions, the complete system evolved a potent activated product (expt. 1). The

TABLE I
THE ACTIVATION OF AHF[a]

Contents of the Second Incubation Mixture	Second Incubation Period (min)	Clotting Time (sec)
1. Act. Christmas factor, AHF	0	220
	10	66
2. Christmas factor, AHF	0	220
	10	186
3. AHF	0	220
	10	220
4. Act. Christmas factor	0	220
	10	220
5. Buffer	0	250
	10	250

[a] The complete reaction mixture in the second incubation mixture contained 0.025 ml of activated-Christmas-factor solution diluted 1:5, 0.4 ml of AHF (2.25 mg protein/ml buffer), 0.025 ml of phospholipid suspension (3.4×10^{-4} M), and 0.05 ml of $CaCl_2$ (0.05 M). Reaction mixtures were incubated at 24° and diluted 1:20 in ice-cold Tris buffer (0.15 M, *p*H 7.5), and an aliquot was assayed immediately for an activated product.

formation of this activated product was dependent upon an initial activation of Christmas factor by activated PTA (expt. 1 versus expt. 2). The small activity evolved when activated PTA was omitted from the first incubation was about 3% of that formed in the complete reaction mixture and was probably owing to some activated PTA present in the crude Christmas factor preparation or to the presence of some activated Christmas factor in the original Christmas-factor preparations. All the other combinations, such as activated PTA and AHF, evolved no clot-accelerating activity.

Figure 1 shows a time curve for the reaction at 24°, wherein two different concentrations of AHF were employed. At this temperature the activated product deteriorated within minutes. No lag phase was observed in this reaction.

Identification of the Activated Product as Activated AHF.—Attempts were then made to relate the initial concentration of AHF added to the final concentration of activated product being formed. When the concentration of activated Christmas factor was held constant and the AHF concentration was doubled, the final concentration of the activated product was a function of the AHF initially added (Fig. 1). In the converse experiment where the AHF concentration was held constant and the concentration of activated Christmas factor was increased, only the rate of forma-

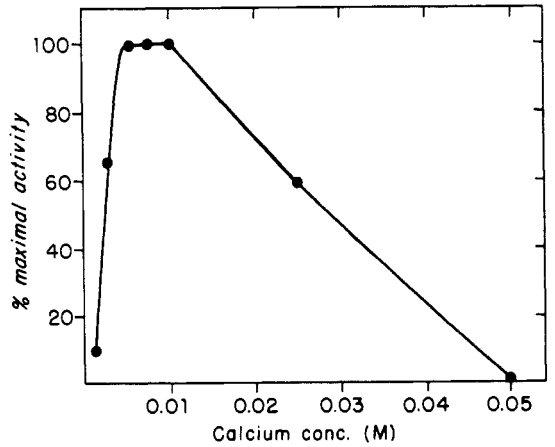

FIG. 2.—The effect of calcium-ion concentration on the initial rate of evolution of activated AHF. Reactions were incubated as described under Table I except that the incubation period was 4 minutes.

TABLE II
EFFECT OF THE ACTIVATED PRODUCT ON VARIOUS HUMAN PLASMAS[a]

Substrate Plasma	Activated Product	Clotting Time (sec) Expt 1	Expt 2
Normal	+	42	54
	−	>250	>250
AHF-deficient	+	48	69
	−	>250	>250
Stuart-deficient	+	240	>350
	−	>250	>350
Proaccelerin-deficient	+	200	220
	−	>250	>250

[a] The reaction mixtures were incubated as described under Table I, but assayed with various substrate plasmas in the presence of calcium ions and phospholipid.

tion of an activated product increased whereas the concentration of the final activated product approached the same final value. These experiments provide strong evidence that activated Christmas factor is an enzyme which converts its substrate, AHF, to an activated form.

To provide further evidence that the activated product was activated AHF and not an activated clotting factor participating in a later stage of coagulation, deficient plasmas were employed for the final assay (Table II). The activated product readily accelerated the clotting of normal plasma as well as that of hemophilic plasma. In contrast, the same fraction had little or no effect on Stuart-deficient or proaccelerin-deficient plasma when it was compared to controls. This provides strong evidence that the activated product formed in our experiments contained little or no activated Stuart factor or activated proaccelerin.

The Effect of Calcium Ions.—Table III shows the activation of AHF in the presence and absence of 0.005 M $CaCl_2$. A marked calcium dependence is observed. The effect of calcium-ion concentration is shown in Figure 2. The optimal calcium-ion concentration is between 5 and 10 mM.

Effect of Phospholipid.—The participation of phospholipid in the clotting scheme follows the activation of Christmas factor by activated PTA (Ratnoff and Davie, 1962). In the present studies, the role of phospholipid was tested by omitting this factor in the incubation reaction which includes activated Christmas factor, AHF, and calcium ions (Table IV). Crude "Centrolex P," which is rich in phospholipid, or a mixture of equal amounts of phosphatidylcholine and phosphatidylserine rapidly accelerate the activation of AHF by activated Christmas factor. At the concentrations tested, phosphatidylserine has about 5% of the maximal activity as compared to the control tube without added phospholipid; phosphatidylcholine shows no activity. The

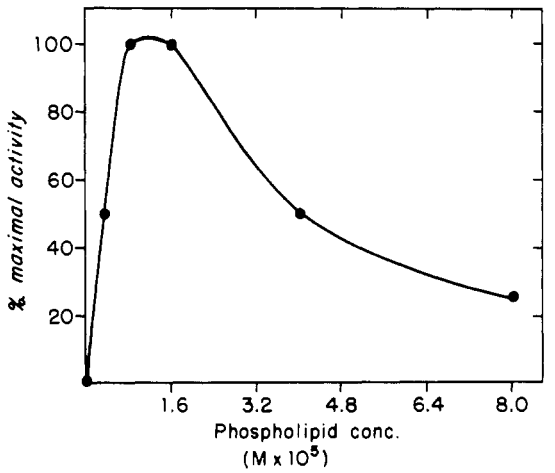

FIG. 3.—The effect of varying the concentration of phospholipid (phosphatidylserine/phosphatidylcholine, 1:1) on the initial rate of evolution of activated AHF. Reactions were incubated as described under Table I except that the incubation period was 5 minutes.

TABLE III
EFFECT OF CALCIUM IONS ON THE ACTIVATION OF AHF BY ACTIVATED CHRISTMAS FACTOR[a]

Additions	Second Incubation Period (min)	Clotting Time (sec)	Per Cent of Control
Calcium ions	0	220	
(5×10^{-3} M)	10	66	100
No calcium added	0	220	
	10	173	4

[a] Reaction mixtures were similar in composition to that listed under Table I. In this experiment with no added calcium, buffer was added in its place.

TABLE IV
EFFECT OF VARIOUS PHOSPHOLIPID FRACTIONS ON THE ACTIVATION OF AHF BY ACTIVATED CHRISTMAS FACTOR[a]

Lipid Fraction Added	Second Incubation Period (min)	Clotting Time (sec) Expt 1	Expt 2	Per Cent of Control
"Centrolex P"	0	160	158	
	4	52	53	100
Phosphatidylcholine + phosphatidylserine	0	145	130	
	4	48	57	100
Phosphatidylcholine	0	180	185	
	4	151	140	4
Phosphatidylserine	0	150	145	
	4	102	103	11
None	0	190	185	
	4	148	167	4

[a] Reaction mixtures were identical in composition to that listed under Table I except for the substitutions or omissions listed above. The reaction mixtures were incubated at 24° for 4 minutes to study initial rates.

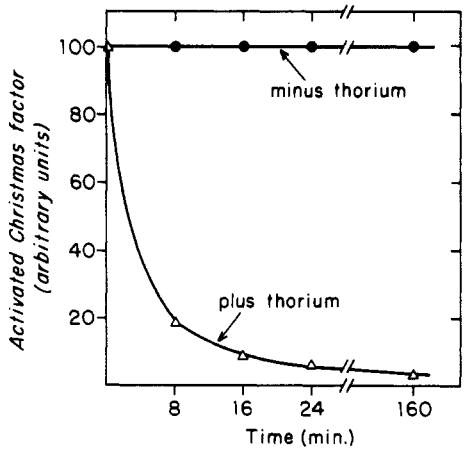

FIG. 4.—The effect of thorium nitrate (3×10^{-4} M) on the coagulant activity of activated Christmas factor. Activated Christmas factor (0.47 ml prepared by the method of White et al. (1953) and 0.03 ml of thorium nitrate (4.8×10^{-3} M) were incubated at 37°. At intervals 0.05-ml aliquots were removed and diluted 1:10 in cold Tris buffer (0.15 M, *p*H 7.5) and assayed. The controls were diluted in the presence of 3×10^{-5} M thorium nitrate.

TABLE V
EFFECT OF PHOSPHOLIPID ON THE CLOT ACCELERATION OF ACTIVATED AHF[a]

Additions to the Substrate Plasma	Second Incubation Period (min)	Clotting Time (sec)	Per Cent of Control
Act. AHF, Ca^{2+}, and "Centrolex P"	0	260	
	6	132	100
	12	112	100
Act. AHF, Ca^{2+}, and phosphatidylserine + phosphatidylcholine	0	260	
	6	168	50
	12	138	49
Act. AHF, Ca^{2+}, and buffer	0	260	
	6	262	
	12	230	

[a] The reaction mixtures were made up and incubated as described under Table I except that the phospholipid concentration (phosphatidylserine, phosphatidylcholine, 1:1) was lowered 5-fold. The activated AHF was diluted 50-fold and assayed in the presence of various phospholipid fractions.

effect of phospholipid concentration employing an equimolar mixture of phosphatidylserine and phosphatidylcholine is shown in Figure 3. The optimal phospholipid concentration occurs at a level of about 1.0×10^{-5} M.

The role of phospholipid in clotting was also tested by adding activated AHF to the substrate plasma and calcium ions in the presence and absence of phospholipid (Table V). In these experiments, the phospholipid added to AHF-activation mixture was lowered 5-fold and the activated product was diluted 50-fold. This greatly reduced the amount of phospholipid transferred to the substrate plasma. It can be seen that phospholipid is required not only in the activation of AHF but in a reaction following the AHF activation. Both the crude "Centrolex P" phospholipid fraction as well as equal mixtures of phosphatidylcholine and phosphatidylserine were active in the final assay. The purified phospholipids, however, appeared to be less active than the crude "Centrolex P" fraction when studied under optimal concentrations.

Inhibition of AHF Activation by Thorium.—Recently, Coleman and Alexander (1964) studied in detail the effects of some lanthanides on blood coagulation. They found that proaccelerin and a new serum factor are progressively inhibited by preincubation with 3×10^{-4} M thorium tetrachloride. Extensive dialysis of the thorium-inhibited factor did not reverse this inhibition. In the present experiments, Christmas factor and activated Christmas factor were incubated with thorium tetranitrate. After removal of this salt by chromatography on Sephadex, the Christmas factor was readily activated by activated PTA and subsequently participated in the activation of AHF. In contrast, activated Christmas factor was markedly inhibited by thorium tetranitrate. Inhibition of activated Christmas factor by 3×10^{-4} M thorium tetranitrate increased progressively with time (Fig. 4). The activity rapidly dropped below 10% of the control after 16 minutes. In these experiments activated Christmas factor was measured directly in the substrate mixture which contained plasma, phospholipid, and calcium ions. A similar inhibition was also noted when activated Christmas factor, preincubated with thorium, was added to AHF in the presence of calcium ions and phospholipid. In the last experiment, the evolution of activated AHF was greatly reduced.

Effect of Other Inhibitors on AHF Activation.—Heparin, at a concentration of 0.025 unit/ml, inhibits the activation of AHF by activated Christmas factor about 50% (Table VI); at a concentration of 0.10 unit/ml, heparin inhibited 100%. In contrast, soybean-trypsin inhibitor (0.05 mg/ml) and DFP had no effect on the system. In the last experiment, activated Christmas factor was preincubated with 1×10^{-3} M DFP at *p*H 7.5 for 60 minutes at 22°, diluted, and added to the AHF, calcium ions, and phospholipid. The control was treated in the same manner except that the DFP was omitted.

TABLE VI
EFFECT OF HEPARIN ON AHF ACTIVATION[a]

Reaction Mixture	Second Incubation Period (min)	Clotting Time (sec) Expt 1	Clotting Time (sec) Expt 2	Per Cent Inhibition
Buffer control	0	162	130	
	5	73	52	
Plus heparin (0.025 unit/ml)	0	135	115	
	5	102	72	50, 50

[a] Reaction mixtures were incubated essentially as described under Table I except that either heparin or buffer were added in addition. Prior to the assay, the buffer controls were diluted in cold Tris buffer containing 0.00125 unit of heparin/ml while the reactions containing heparin were diluted in buffer alone.

DISCUSSION

The present experiments employing partially purified reagents provide strong evidence that AHF in the presence of calcium ions and phospholipid is converted to an activated form by activated Christmas factor (equation 4).

$$\text{AHF} \xrightarrow[Ca^{2+},\ \text{phospholipid}]{\text{act. Christmas factor}} \text{activated AHF} \qquad (4)$$

The evidence shows that activated Christmas factor participates as an enzyme and AHF as the substrate; no lag phase was observed in this reaction. As shown in Table I, AHF activation is reduced about 95%

when Christmas factor is employed rather than activated Christmas factor.

In these experiments, the ratio of activated Christmas factor to AHF was carefully adjusted so that the reaction went to completion within 10 minutes. This is important since the activated AHF is labile. Also, calcium and phospholipid appear to form slowly a complex which apparently is inhibitory to activated Christmas factor. Thus reactions requiring an incubation period greater than 10 minutes usually do not show the proper substrate-product relationship. Excessive activated Christmas factor relative to the amount of AHF also must be avoided. Preliminary results indicate that the substrate for activated AHF is present in the activated-Christmas-factor preparation. Thus, with high Christmas factor concentrations, the concentration of the final activated product (or products) will no longer be proportional to the AHF initially added. This can be readily shown when the ratio of AHF to activated Christmas factor is decreased about 50-fold and a more stable activated product is formed. The nature of the clotting contaminant in the Christmas-factor preparation which is a potential substrate for activated AHF has not been established but preliminary experiments suggest that it is probably Stuart factor.

A reaction involving AHF which took place in the absence of phospholipid was studied by Biggs and Bidwell (1959). They observed a marked lag phase in the formation of an activated product. This lag phase may be due to a preliminary activation of Christmas factor by activated PTA prior to the participation of AHF. In our experiments, considerably more Christmas factor is required when Christmas factor is not completely activated and phospholipid is omitted and the problem of interference from contaminating clotting factors is greatly increased. Under such conditions, the final activated product appears to resemble product I of Bergsagel and Hougie (1956). This enzyme apparently is identical to activated Stuart factor (Spaet and Cintron, 1963).

Evidence for a phospholipid requirement for AHF activation as well as in a stage following AHF activation has been presented. The requirement for phospholipid for AHF activation is consistent with the findings that phospholipid accelerates the formation of product I (C. Hougie, personal communication). A phospholipid requirement in the late stages of coagulation recently has been shown to occur during the activation of proaccelerin by activated Stuart factor (R.T. Breckenridge and O.D. Ratnoff, unpublished results). The phospholipid requirement in both steps can be satisfied by a mixture of phosphatidylserine and phosphatidylcholine. These findings are consistent with the earlier findings on the phospholipid requirement (Marcus and Spaet, 1958; Troup *et al.*, 1958; Papahadjopoulos *et al.*, 1962). Reasons for the increased activity in the latter phase of coagulation by crude "Centrolex P" phospholipid as compared to the phosphatidylserine-phosphatidylcholine mixture will require further studies. Possible change in phospholipid components during AHF activation has not been investigated. In our experiments, calcium and phospholipid were added separately at zero time; the mechanism of the inhibitory effects of phospholipid preincubated with calcium requires further investigation.

The inhibition of activated Christmas factor by thorium salts adds to the list of thorium-sensitive clotting factors. It appears likely that the thorium-vulnerable factor described by Colman and Alexander (1964) is not related to activated Christmas factor since, as these authors point out, serum prepared from Christmas-deficient plasma readily corrects the thorium-treated serum.

The lack of inhibition of the activated-Christmas-factor preparation by DFP provides further evidence that the present system is not related to the thrombin activation of AHF described by Rapaport *et al.* (1963). If thrombin were participating, it would have been blocked by DFP and little or no AHF activation would have occurred. The requirement for Christmas-factor activation by activated PTA prior to AHF activation is also inconsistent with the participation of thrombin in the present system.

The inhibition of AHF activation by heparin is consistent with the earlier observations of O'Brien (1958, 1960), Schanberge *et al.* (1959), and Greig (1959), which indicated that heparin inhibits the action of Christmas factor in thromboplastin generation. Thus, heparin not only blocks Christmas-factor activation by activated PTA (Ratnoff and Davie, 1962), but also Christmas factor after it is activated.

The mechanism by which the activation of AHF occurs is unknown but it appears likely that some rearrangement of the AHF molecule occurs unmasking or forming a catalytic site. Studies on the differences of AHF and activated AHF are now in progress.

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References

Addis, T. (1911), *J. Pathol. Bacteriol. 15*, 427.
Alexander, B. (1955), *in* The Coagulation of Blood, Methods of Study, Tocantins, L. M., ed., New York, Grune & Stratton.
Bergsagel, D. E., and Hougie, C. (1956), *Brit. J. Haematol. 2*, 113.
Biggs, R., and Bidwell, E. (1959), *Proc. Intern. Congr. Biochem. 4th Vienna, 1958, 10*, 172.
Breckenridge, R. T., and Ratnoff, O. D. (1962), *Blood 20*, 137.
Brinkhous, K. M. (1939), *Am. J. Med. Sci. 198*, 509.
Coleman, R., and Alexander, B. (1964), *J. Clin. Invest. 43*, 705.
Fisch, U., and Duckert, F. (1959), *Thromb. Diath. Haemorrhag. 3*, 98.
Gornall, A. G., Bardawill, C. J., and David, M. M. (1949), *J. Biol. Chem. 177*, 751.
Greig, H. B. W. (1959), *Lancet 2*, 25.
Marcus, A. J., and Spaet, T. H. (1958), *J. Clin. Invest. 37*, 1836.
O'Brien, J. R. (1958), *Nature 181*, 1801.
O'Brien, J. R. (1960), *J. Clin. Pathol. 13*, 93.
Papahadjopoulos, D., Hougie, C., and Hanahan, D. J. (1962), *Proc. Soc. Exptl. Biol. Med. 111*, 412.
Rapaport, S. I., Schiffman, S., Patch, M. J., and Ames, S. B. (1963), *Blood 21*, 221.
Ratnoff, O. D., and Davie, E. W. (1962), *Biochemistry 1*, 677.
Ratnoff, O. D., Davie, E. W., and Mallett, D. L. (1961), *J. Clin. Invest. 40*, 803.
Schanberge, J. N., Sarelis, A., and Regan, E. E. (1959), *J. Lab. Clin. Med. 54*, 501.
Spaet, T. H., and Cintron, J. (1963), *Blood 21*, 745.
Troup, S. B., Reed, C. F., Marinetti, G. V., and Swisher, S. N. (1958), *J. Clin. Invest. 39*, 342.
White, S. G., Aggelar, P. M., and Glendening, M. B. (1953), *Blood 8*, 101.