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The Activation of Stuart Factor (Factor X) by Activated Antihemophilic Factor (Activated Factor VIII)*

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ABSTRACT: The reaction of activated antihemophilic factor with Stuart factor has been investigated. It was found that activated antihemophilic factor is an enzyme which reacts with Stuart factor converting the latter to an activated product which accelerates clotting. The reaction occurs only in the presence of calcium ions, the optimal concentration being 5 mM. The *p*H optimum was found to be about 7.5. The activation of Stuart factor was not affected by 1×10^{-4} M *p*-mercuribenzoate or 1.0 unit of heparin per ml. Esterase activity toward benzoylarginyl ethyl ester or tosylarginymethyl ester was not detected for either activated antil hemophilic factor or activated Stuart factor. In addition, neither enzyme was inhibited by preincubation with 5×10^{-3} M diisopropylphosphorofluoridate. Soybean trypsin inhibitor was found to be a potent inhibitor of activated Stuart factor. Phospholipid and calcium ions were required for coagulation at some point(s) following the activation of Stuart factor in the intrinsic system.

Stuart factor[1] (factor X) is a plasma protein which is required for normal blood coagulation (Telfer *et al.*, 1956; Hougie *et al.*, 1957). In the intrinsic clotting system, it participates during the middle phase of blood coagulation following the interaction of Hageman factor (factor XII), plasma thromboplastin antecedent (factor XI), and Christmas factor (factor IX) (see Davie and Ratnoff, 1964, and Macfarlane, 1964, for recent reviews). It has been shown that Stuart factor is converted to an activated form (product I) in the presence of activated Christmas factor, antihemophilic factor (factor VIII), and calcium ions (Bergsagel and Hougie, 1956; Hougie *et al.*, 1957; Spaet and Cintron, 1963). Lundblad and Davie (1964) have shown that the initial reaction involving the last three clotting factors is the activation of antihemophilic factor by activated Christmas factor. This reaction requires the presence of phospholipid and calcium ions. The present communication characterizes the second reaction in which activated antihemophilic factor in turn converts Stuart factor to an activated form in a calcium-dependent reaction.

Materials and Methods

Standard grade *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., Piscataway, New Market, N.J. Crystalline *soybean trypsin inhibitor* (STI)[2] was purchased from

* From the Department of Biochemistry, University of Washington, School of Medicine, Seattle. *Received September 17, 1964*. This study was supported in part by a research grant (GM 10795-02) from the National Institutes of Health, and by State of Washington Initiative 171 Funds for Research in Biology and Medicine. A portion of this work was conducted through the Clinical Research Center Facility of the University of Washington supported by the National Institutes of Health.

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[1] Stuart factor was named after one of the earliest patients in which a deficiency of this clotting factor was observed (Hougie *et al.*, 1957).

[2] Abbreviations used in this work: STI, soybean trypsin inhibitor; DFP, diisopropylphosphorofluoridate; TAME, *p*-toluenesulfonyl-L-arginine methyl ester; BAEE, benzoyl-L-arginine ethyl ester; AHF, antihemophilic factor; PTA, plasma thromboplastin antecedent.

Worthington Biochemical Corp., Freehold, N.J. Sodium *heparin* (1000 USP units/ml) was purchased from Invenex, San Francisco, Calif. *Diisopropylphosphorofluoridate* (DFP) was purchased from K and K Laboratories, Long Island City, N.Y. *p-Mercuribenzoate* was purchased from Sigma Chemical Co., St. Louis, Mo. Rabbit brain *thromboplastin* was purchased from Difco Laboratories, Detroit, Mich. It was prepared for use by suspending the thromboplastin in 0.15 M NaCl (37 mg/ml) and warming the mixture at 45° for 10 minutes according to the manufacturer's instruction. Bovine *fibrinogen* was purchased from Warner-Chilcott, Morris Plains, N.J. *p-Toluenesulfonyl-L-arginine methyl ester* (TAME) was purchased from H. M. Chemical Co., Ltd., Santa Monica, Calif. *Benzoyl-L-arginine ethyl ester* (BAEE) was synthesized by the procedure of Bergmann *et al.* (1939).

Crude phospholipid (Centrolex P) was purchased from Central Soya, Chicago, Ill. A 0.10% suspension was prepared by homogenization of crude phospholipid in 0.15 M NaCl in a Waring Blendor. Aliquots were stored in Lusteroid tubes at −20°. Phosphatidylserine and phosphatidylcholine were kindly provided by Dr. D. Papahadjopoulos, Dr. D. J. Hanahan, and Mr. C. Jackson, in an emulsified form at a concentration of 1.7×10^{-3} M. The preparation and properties of these compounds have been described elsewhere (Papahadjopoulos *et al.*, 1962). Bovine *antihemophilic factor* (AHF) was purchased from S. Maw and Sons, Ltd., Barnet, England. *Activated AHF* was prepared immediately prior to use by the method of Lundblad and Davie (1964). *Activated plasma thromboplastin antecedent* (activated PTA) was prepared from human serum by the method of Ratnoff and Davie (1962).

Crude *Stuart factor* was obtained from bovine prothrombin-poor serum. The serum was absorbed with $BaSO_4$ (Baker) at a concentration of 100 mg/ml of serum for 15 minutes at room temperature with constant stirring. The suspension was centrifuged to collect the $BaSO_4$ precipitate. The $BaSO_4$ was then washed twice with one-half the original volume of cold 0.15 M NaCl. After the second wash the Stuart factor activity was eluted by suspending the $BaSO_4$ precipitate in one-half the original volume of 10% (w/v) trisodium citrate dihydrate solution and mixing for 10 minutes at room temperature. The $BaSO_4$ was then removed by centrifugation and the supernatant containing Stuart factor activity was stored in 10-ml aliquots at −20°. Prior to use the crude Stuart factor was thawed and dialyzed against 500 volumes of 0.15 M NaCl in the cold for 12 hours. The dialyzed crude Stuart factor had a protein concentration of 2.6–3.4 mg/ml and contained measurable amounts of Christmas factor and factor VII.

Crude *Christmas factor* was prepared from prothrombin-poor bovine serum by the method of Lundblad and Davie (1964).

Platelet-deficient citrated *human plasma* was prepared in silicone-coated glassware from blood to which one-ninth volume of 0.13 M trisodium citrate had been added (Ratnoff and Davie, 1962). This plasma, which had no direct contact with a glass surface, was stored at −20°.

Human plasmas deficient in AHF, Stuart factor, and proaccelerin were kindly provided by Drs. C. Hougie, L. W. Gaston, and O. D. Ratnoff. The plasma deficient in AHF and Stuart factor were fortified with traces of $BaSO_4$-adsorbed bovine serum since these plasmas were low in proaccelerin owing to their age. Assays for AHF, Stuart factor, and proaccelerin were done by previously published methods (Ratnoff *et al.*, 1961; Breckenridge and Ratnoff, 1962).

Protein was determined by the biuret procedure of Gornall *et al.* (1949). A freshly prepared solution of crystalline bovine serum albumin was used as a standard.

The *activation of Stuart factor* was studied by incubating 0.05 ml activated AHF containing 0.15 mg of protein, 0.3 ml of crude Stuart factor containing 0.8 mg of protein, 0.05 ml of 0.05 M $CaCl_2$, and 0.10 ml of 0.15 M Tris buffer, *p*H 7.5, at 24°. At intervals 0.05-ml aliquots of the incubation mixture were removed and diluted 1 : 20 in 0.15 M Tris buffer, *p*H 7.5, at 0°. Aliquots (0.1 ml) of the diluted activation mixture were immediately assayed by their addition to 0.1 ml substrate plasma, 0.1 ml 0.025 M $CaCl_2$, and 0.1 ml 0.1% Centrolex P. The clotting time was then determined at 37°. Because of the dilution of the activation mixtures, the concentrations of the reactants during the assay step were kept much lower than in the activation step. This helped to minimize additional activation of Stuart factor during the final assay. It also lowered the zero-time value for the activation reaction which was caused primarily by the presence of activated AHF. The zero-time blank gave a clotting time ranging from 90–150 seconds depending on the level of activated AHF initially present in the activation reaction.

The degree of activation of Stuart factor was determined from a standard curve prepared from the sample with the greatest activity. This sample was diluted to several concentrations in cold 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 sample with maximal activity was arbitrarily expressed as 100 units of activated Stuart factor.

Esterase activity toward BAEE and TAME was performed by a titrimetric method previously described (Schwert *et al.*, 1948).

The presence of *thrombin* was determined by the addition of a 0.1-ml test sample to 0.2 ml of bovine fibrinogen solution containing 3.0 mg coagulable protein per ml. The above solutions were quickly mixed and the tube, immersed in a 37° bath, was tipped every 50 seconds until the formation of a clot was observed.

Results

Formation of an Activated Product. The incubation of activated AHF and calcium ions with a crude Stuart

TABLE I: Formation of an Activated Product.[a]

Contents of Activation Reaction	Incubation Time for Activation Reaction (min)	Clotting Time (sec) Expt 1	Expt 2
Activated AHF +	0	91	130
crude Stuart factor	10	50	50
Activated AHF	0	109	160
	10	120	175
Crude Stuart factor	0	180	205
	10	161	196
AHF +	0	150	220
crude Stuart factor	10	130	160

[a] The complete system contained 0.05 ml activated AHF containing 0.15 mg of protein, 0.3 ml crude Stuart factor containing 0.80 mg of protein, 0.05 ml of 0.05 M $CaCl_2$, and 0.10 ml of 0.15 M Tris buffer, *p*H 7.5. The reactants were incubated for 10 minutes at 24°. At the given time intervals 0.05-ml aliquots were removed, diluted 1:20 in cold 0.15 M Tris buffer, *p*H 7.5, and immediately assayed for clot-promoting activity.

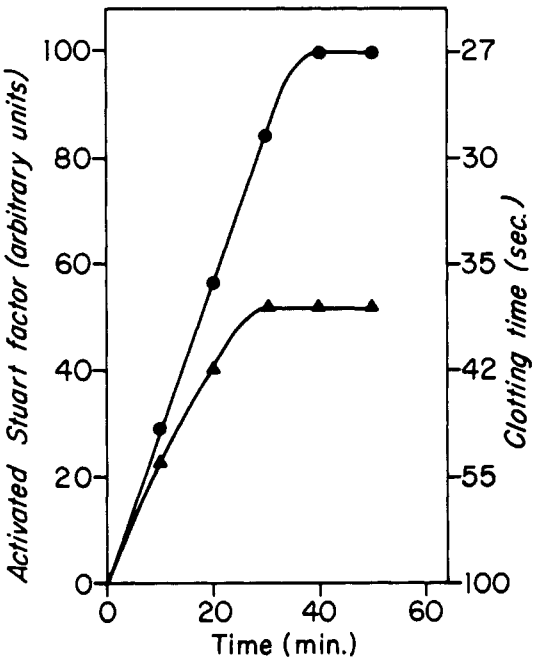


FIGURE 1: The formation of activated Stuart factor with time at two different concentrations of Stuart factor. Reactions were incubated as described under Table I. ●—● contained 0.6 mg Stuart factor/ml and ▲—▲ contained 0.3 mg Stuart factor/ml.

factor preparation gave rise to a potent activated product (Table I). This clot-accelerating activity was approximately 10–15 times greater than that observed at zero time, i.e., dilution of the final activated product 10- to 15-fold produced the same clotting time as that at zero time. The formation of the activated product was dependent on the presence of crude Stuart factor and activated AHF. AHF which was not previously activated with activated Christmas factor showed little activity.

Identification of the Activated Product as Activated Stuart Factor. A time curve showing the formation of the activated product at two different concentrations of crude bovine Stuart factor is seen in Figure 1. The concentration of activated AHF was held constant in these experiments. The formation of the activated product leveled off in both cases after about 40 minutes, the final activity being proportional to the amount of crude Stuart factor initially added. In the converse experiment, the crude bovine Stuart factor concentration was held constant and the concentration of activated AHF was doubled (Figure 2). The rate of formation of activated product increased about 2-fold with twice the concentration of activated AHF, whereas the concentration of the final product remained constant. These experiments provide strong evidence that activated AHF is an enzyme which converts its substrate, Stuart factor, to an activated form.

The substrate of activated AHF was further identified as Stuart factor by employing crude Stuart factor preparations from normal and Stuart-deficient serum. The results of these experiments are shown in Figure

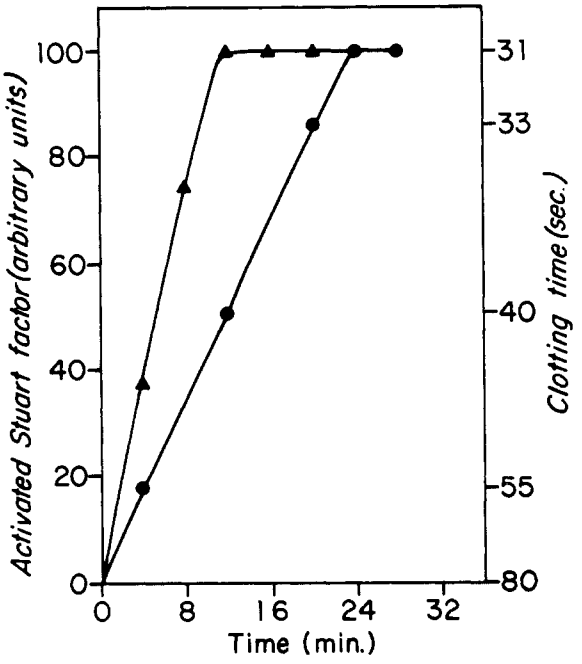


FIGURE 2: The formation of activated Stuart factor with time at two different concentrations of activated AHF. Reactions were incubated as described under Table I. ▲—▲ contained 0.3 mg activated AHF/ml and ●—● contained 0.15 mg activated AHF/ml.

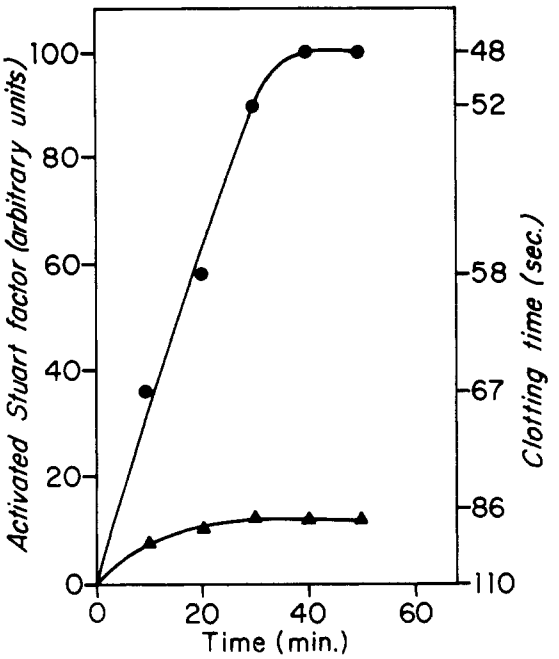


FIGURE 3: The formation of activated Stuart factor with time. Stuart factor was prepared from normal human serum and Stuart-deficient serum. Reactions were carried out as described under Table I. $BaSO_4$ eluates, prepared in an identical manner containing the same amount of protein from normal and deficient serum, were used as a source of crude Stuart factor. Aliquots of the various incubation mixtures were removed, diluted 1 : 20 in cold 0.15 M Tris buffer, *p*H 7.5, and assayed immediately for clot-promoting activity with normal substrate plasma. ●—●, Stuart factor prepared from normal serum; ▲—▲, Stuart factor prepared from deficient serum.

3. Crude Stuart factor prepared from normal human serum evolves a potent activated product with time similar to that observed with crude bovine Stuart factor. In contrast, crude Stuart factor preparations from Stuart-deficient serum yield only about one-tenth as much activated product.

To provide further evidence that the activated product was activated Stuart factor and not an activated clotting factor participating in a later known stage of coagulation, deficient plasmas were employed for the final assay. As shown in Table II, the activated product readily accelerated the clotting of normal human plasma as well as that of hemophilic plasma and Stuart-deficient plasma. The activated product had little or no effect on proaccelerin-deficient plasma when it was compared to controls.

These experiments provide additional evidence that the activated product formed was activated Stuart factor and contained little or no activated proaccelerin. The activated Stuart factor, when assayed at 20-fold greater concentration than that used for the above experiments, contained no detectable thrombin activity.

TABLE II: Effect of the Activated Stuart Factor on Various Human Plasmas.[a]

Substrate Plasma	Activated Product	Clotting Time (sec) Expt 1	Expt 2
Normal	+	33	41
	−	>250	>250
AHF deficient	+	41	45
	−	>250	>250
Stuart deficient	+	53	49
	−	>250	>250
Proaccelerin deficient	+	195	250
	−	>250	>250

[a] Activated Stuart factor was prepared as described under Table I. Aliquots were removed and diluted 1:20 in cold 0.15 M Tris buffer, *p*H 7.5. The diluted activated Stuart factor (0.1 ml) was added to 0.1 ml substrate plasma, 0.1 ml 0.025 M $CaCl_2$, and 0.1 ml 0.1% Centrolex P. The clotting time was then determined at 37°. In the controls, buffer was added in place of the activated product.

TABLE III: Effect of Calcium Ions on Stuart Factor Activation.[a]

Additions	Incubation Time (min)	Clotting Time (sec) Expt 1	Expt 2
5×10^{-3} $CaCl_2$	0	109	129
	10	58	58
Buffer control	0	155	160
	10	192	195

[a] The incubations were carried out as described under Table I. Aliquots were removed from the incubation mixtures, diluted 1:20 in cold 0.15 M Tris buffer, *p*H 7.5, and assayed for clot-promoting activity with normal substrate plasma in the presence of calcium ions and phospholipid at 37°.

Effect of Divalent Cations. Table III shows the activation of Stuart factor in the presence and absence of 0.005 M $CaCl_2$. A marked calcium ion dependence is observed. The effect of calcium ion concentration is shown in Figure 4. The optimal calcium ion concentration is about 5 mM. Other cations such as Ba^{2+}, Cu^{2+}, Cd^{2+}, Co^{2+}, Mg^{2+}, Mn^{2+}, Sr^{2+}, Zn^{2+}, and Ni^{2+} were inactive when tested as their chlorides in the concentration range of 5×10^{-4} to 1×10^{-2} M.

*Effect of p*H. The effect of *p*H on the initial rate of formation of activated Stuart factor is shown in Figure 5. The *p*H optimum for the reaction is about 7.5.

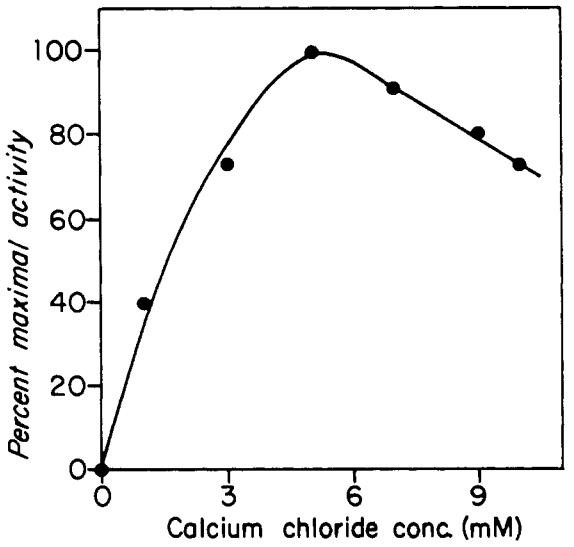


FIGURE 4: The effect of calcium ion concentration on the initial rate of activation of crude Stuart factor by activated AHF. The reactions were carried out as described under Table I. Solutions of various $CaCl_2$ concentrations were used as the source of calcium ions. Aliquots were removed at zero time and at 10 minutes, diluted 1:20 in cold 0.15 M Tris buffer, *p*H 7.5, and assayed immediately using normal substrate plasma.

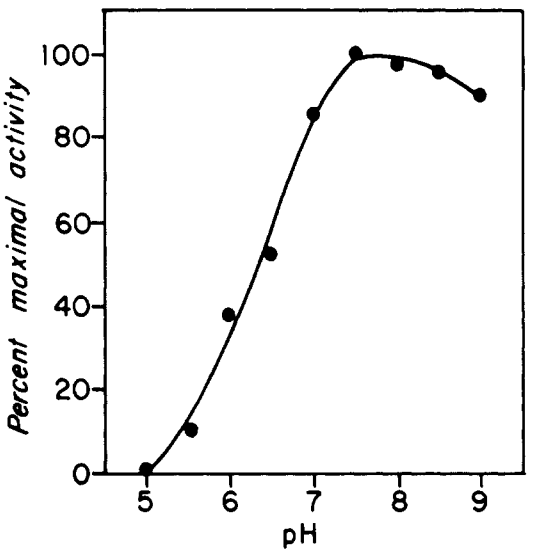


FIGURE 5: The effect of *p*H on the initial rate of activation of crude Stuart factor by activated AHF. Buffers were composed of 0.05 M Tris–0.05 M imidazole–0.5 M acetate adjusted to the various *p*H values by the addition of hydrochloric acid or sodium hydroxide. Aliquots were removed at zero time and at 10 minutes, diluted 1:20 in cold 0.15 M Tris buffer, *p*H 7.5, and assayed immediately for clot-promoting activity with normal substrate plasma.

Esterase Activity of Activated Stuart Factor. Activated Stuart factor has been reported to possess esterase activity toward TAME (Esnouf and Williams, 1962; Milstone, 1962) although Zucker-Franklin and Spaet (1963) found no esterase activity for their partially purified product I. In the present experiments, activated Stuart factor was incubated with either 0.01 M TAME, *p*H 8.4, or 0.01 M BAEE, *p*H 8.4, for 20 minutes at a concentration twenty times that required to clot normal plasma in 30 seconds. In these experiments, the ester assay contained 0.05 M $CaCl_2$, 0.01 M Tris buffer, *p*H 8.4, 0.10 M KCl, and substrate. No ester hydrolysis above the blanks occurred with activated Stuart factor. Preliminary experiments with activated AHF gave similar negative results with the same ester substrates.

Effect of Various Inhibitors. Previous studies have shown the effect of a number of inhibitors on specific steps in blood coagulation. Thus DFP has been shown to be a potent inhibitor of activated plasma thromboplastin antecedent (Ratnoff and Davie, 1962; Kingdon *et al.*, 1964) and thrombin (Gladner and Laki, 1958). Likewise, heparin has been shown to inhibit the activation of Christmas factor (Ratnoff and Davie, 1962), the activation of AHF (Lundblad and Davie, 1964), and the interaction of the thrombin-fibrinogen system (Klein and Seegers, 1950; Godal, 1961). STI has been shown to block activated Stuart factor prepared with Russell's viper venom (Breckenridge and Ratnoff, 1964). Thus, it was of interest to test these various blood coagulation inhibitors in the present reaction for similar effects.

It was found that the activation of Stuart factor was not affected by 1×10^{-4} M *p*-mercuribenzoate or 1.0 unit of heparin per ml. Furthermore, preincubation of either activated AHF or activated Stuart factor with 5×10^{-3} MDFP for 5 minutes at 25° caused no detectable loss in enzymatic activity when compared with controls. The same DFP preparation readily inactivated thrombin under similar conditions.

STI, however, was found to be a potent inhibitor of activated Stuart factor (Table IV). In these experiments, STI was added to activated Stuart factor and the resulting mixture was diluted 20-fold in buffer at 0° and assayed in the usual manner. The control was diluted in buffer containing 1 μg of STI per ml. Thus the final assay mixture contained the same concentration of inhibitor for both experiments. Since STI reacted so strongly with activated Stuart factor, it was not possible to test it as a potential inhibitor of the activation of Stuart factor by activated AHF.

The Effects of Phospholipid and Calcium Following Stuart Factor Activation. Preliminary results indicated that the addition of phospholipid had no effect on the activation of Stuart factor by activated AHF. Phospholipid is required, however, following Stuart factor activation. A similar absolute requirement is observed for calcium ions (Table V). In these experiments either phospholipid or calcium ions were omitted in the substrate plasma to which activated Stuart factor was added.

TABLE IV: Effect of Soybean Trypsin Inhibitor on Activated Stuart Factor.[a]

Addition	Clotting Time (sec) Expt 1	Expt 2
STI (20 μg/ml)	83	75
Buffer	36	34

[a] Crude activated Stuart factor (0.45 ml) prepared as described under Table I, was mixed with 0.05 ml of STI (0.2 mg/ml in 0.15 M Tris, *p*H 7.5) and aliquots were diluted 1:20 in cold 0.15 M Tris buffer, *p*H 7.5, and assayed for clot-promoting activity with normal substrate plasma in the presence of phospholipid and calcium ions. In the buffer control 0.05 ml of 0.15 M Tris buffer, *p*H 7.5, replaced the volume of STI solution. Aliquots of the control were diluted 1:20 in cold 0.15 M Tris buffer, *p*H 7.5, containing 1 μg STI/ml, and assayed as described above.

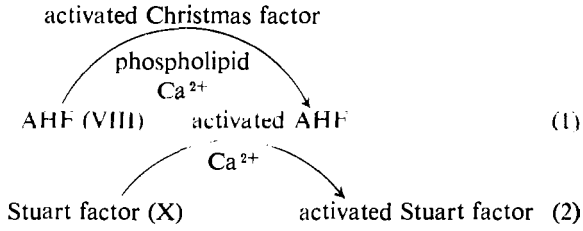
TABLE V: Effect of Calcium Ions and Phospholipid on the Clot-Acceleration of Activated Stuart Factor.[a]

Additions to the Substrate Plasma	Clotting Time (sec) Expt 1	Expt 2
Activated Stuart factor, Ca^{2+}, and phospholipid	33	23
Activated Stuart factor, Ca^{2+}, and 0.15 M NaCl	94	95
Activated Stuart factor, phospholipid, and buffer	>200	>250
Activated Stuart factor and buffer	>200	>250

[a] Crude activated Stuart factor was prepared as described under Table I. Aliquots were diluted 1:20 in cold 0.15 M Tris buffer, *p*H 7.5, and assayed immediately. In the last three experiments, either phospholipid or calcium ions or both were omitted in the substrate plasma. The clotting times of the various substrate mixtures were determined at 37°.

Discussion

The present results provide strong evidence that activated AHF is an enzyme which, in the presence of calcium ions, is capable of converting its substrate, Stuart factor, to an activated form. This reaction follows the activation of AHF by activated Christmas factor, a reaction which occurs in the presence of phospholipid and calcium ions (Lundblad and Davie, 1964). These two closely related reactions are shown in equations (1) and (2).



As we have previously emphasized, these conclusions are based upon experiments employing preparations of crude clotting factors. Thus, it is possible that other clotting factors, not detected in the present experiments, may participate between activated Christmas factor and Stuart factor. The use of Stuart deficient serum as a source of Stuart factor however supports the conclusion that Stuart factor is the substrate for activated AHF. In these experiments, the concentration of activated product was reduced about 10-fold as compared to the normal human serum controls prepared in an identical manner. This is similar to the actual level of Stuart factor in deficient plasma. The experiments substituting deficient plasmas in the assay for activated product are also consistent with the conclusion that Stuart factor is the substrate for activated AHF. Activated Stuart factor accelerates the clotting of Stuart-deficient plasma but not of proaccelerin-deficient plasma. Thus the activated Stuart factor is not contaminated with other activated clotting factors such as activated proaccelerin or thrombin, which participate in the final phases of coagulation.

Previously (Lundblad and Davie, 1964) we observed that activated AHF deteriorated rapidly at 25°. As shown in Figure 1, however, the stability of AHF was markedly increased in the presence of its substrate. These results suggest that Stuart factor should be converted in high yield to activated Stuart factor during normal coagulation. This, however, is not the case since Stuart factor is found at the end of coagulation, i.e., in serum, primarily in its precursor form. Indeed, Macfarlane and Ash (1964) have found that the addition of excess AHF to normal plasma produced significantly more activated Stuart factor than did the control plasma following recalcification and clotting. Thus, it appears probable that other reactions such as thrombin destruction of AHF (Penick, 1957; Surgenor *et al.*, 1961) or activated AHF may occur, the availability of phospholipid may be limited, or certain inhibitors of Stuart factor activation may arise during coagulation and consequently limit the activation of Stuart factor under physiological conditions. Such reactions could serve as control mechanisms of coagulation.

The activation of Stuart factor by activated AHF is analogous to its activation by Russell's viper venom (Esnouf and Williams, 1962; Macfarlane, 1962), trypsin (Alexander *et al.*, 1962; Papahadjopoulos *et al.*, 1965), cathepsin C (Purcell and Barnhart, 1963), papain (Alexander *et al.*, 1962), and a complex of factor VII and tissue thromboplastin (Nemerson and Spaet,

1964; Williams, 1964). The activation by Russell's viper venom apparently occurs via partial hydrolysis, for a second component appears upon electrophoresis and ultracentrifugation and new N-terminal amino acids appear (Esnouf and Williams, 1962). A reduction in molecular size and a sharp decrease in the net negative charge following activation by Russell's viper venom or trypsin was also noted by Papahadjopoulos *et al.* (1965). Whether Stuart factor activation by activated AHF occurs via an analogous manner will require further study.

The calcium dependence for Stuart factor activation is the third consecutive reaction in the intrinsic system requiring a divalent metal. Previous studies have shown a calcium requirement for Christmas factor activation (Ratnoff and Davie, 1962) and AHF activation (Lundblad and Davie, 1964). The Stuart factor activation, however, is the only one having an absolute requirement for Ca^{2+}. In the activation of Christmas factor, Sr^{2+}, Cu^{2+}, and Zn^{2+} ions are active (Kingdon and Davie, 1965) while Sr^{2+} and Mn^{2+} are active in the conversion of AHF to activated AHF (R. Lundblad and E. W. Davie, unpublished data).

A requirement for calcium following the activation of Stuart factor was also demonstrated. R. T. Breckenridge and O. D. Ratnoff recently (unpublished results) have observed a calcium dependence for the reaction between activated Stuart factor and proaccelerin employing human preparations. With bovine preparations the formation of a stable complex between activated Stuart factor, proaccelerin, and phospholipid has been observed by gel filtration only in the presence of calcium ions (Papahadjopoulos and Hanahan, 1964). Preliminary data suggest that calcium ions greatly potentiate the action of activated proaccelerin on prothrombin (Breckenridge and Ratnoff, unpublished results). Calcium also stimulates the thrombin-fibrinogen interaction about 2-fold (Seegers and Smith, 1942; Ratnoff and Potts, 1954), but its presence is not an absolute requirement. Under the present experimental conditions, the calcium dependent reactions involving the fibrin-stabilizing factor would not affect the assay. Thus, the effects of calcium following the activation of Stuart factor appear to be related to several additional steps in coagulation.

A phospholipid requirement following the activation of Stuart factor was also observed. These findings are similar to those of Peden and Peacock (1958), Macfarlane (1961), Papahadjopoulos *et al.* (1962), Breckenridge and Ratnoff (1963), and Zucker-Franklin and Spaet (1963). This requirement is associated with the activation of proaccelerin by activated Stuart factor (R. D. Breckenridge and O. D. Ratnoff, unpublished results).

In the present studies, DFP had no inhibitory effect on activated Stuart factor. Furthermore, at the concentrations of activated Stuart factor tested, esterase activity was not detected. These findings are similar to the results of Zucker-Franklin and Spaet (1963) who characterized product I as a coagulant arising from Stuart factor (Spaet and Cintron, 1963). The former workers found neither esterase activity toward benzoyl-L-arginine methyl ester nor DFP sensitivity for product I.

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Enzymatic Synthesis of Analogs of the Cell-Wall Precursor. I. Kinetics and Specificity of Uridine Diphospho-*N*-acetylmuramyl-L-alanyl-D-glutamyl-L-lysine:D-Alanyl-D-alanine Ligase (Adenosine Diphosphate) from *Streptococcus faecalis* R*

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ABSTRACT: A specificity profile of the enzyme uridine diphospho-*N*-acetylmuramyl-L-alanyl-D-glutamyl-L-lysine:D-alanyl-D-alanine ligase (ADP) has been established. The addition of substituents to the N-terminal residue of the dipeptide enhances the binding of dipeptide in two cases. In contrast, the addition of substituents to the C-terminal residue decreases the binding of the dipeptide in every case. For example, the following pattern is observed ($K_{m_t} \times 10^4$): D-α-amino-*n*-butyryl-D-alanine (0.9), D-norvalyl-D-alanine (1.2), D-alanyl-D-alanine (1.6), D-alanyl-D-α-amino-*n*-butyryic acid (7.6), D-alanyl-D-norvaline (>50). The kinetic studies are consistent with a uridine nucleotide and dipeptide binding site which are independent of each other. A comparison of the specificity profile of this enzyme with that of D-alanine–D-alanine ligase (ADP) has established a cooperative specificity pattern between two sequential enzyme reactions which are involved in the incorporation of D-alanine into the bacterial cell-wall precursor, UDP-NAc-muramyl-L-Ala-D-Glu-L-Lys-D-Ala-D-Ala.

The basal structure of many bacterial cell walls includes a peptide containing L-alanine, D-glutamic acid, L-lysine (or diaminopimelic acid), and D-alanine covalently linked to muramic acid (3-*O*-lactyl-NAc-glucosamine) (Salton, 1964). The precursor of the NAc-muramyl-peptide moiety is believed to be UDP-NAc-muramyl-L-Ala-D-Glu-L-Lys-D-Ala-D-Ala (Strominger *et al.*, 1959a). This is the predominant nucleotide which accumulates when many bacteria are exposed to the antibiotic penicillin (Park, 1952a,b,c; Strominger, 1957; Saito *et al.*, 1963). It is formed by the addition of amino acids to UDP-NAc-muramic acid, catalyzed by the sequential action of four enzymes (Ito and Strominger, 1962a,b, 1964; Nathenson *et al.*, 1964). They are as follows: (1) UDP-NAc-muramic acid:L-alanine ligase (ADP), (2) UDP-NAc-muramyl-L-Ala:D-glutamic acid ligase (ADP), (3) UDP-NAc-muramyl-L-Ala-D-Glu:L-lysine ligase (ADP) (6.3.2.7), (4) UDP-NAc-muramyl-L-Ala-D-Glu-L-Lys:D-Ala-D-Ala ligase (ADP) (Comb, 1962). The synthesis of the dipeptide D-Ala-D-Ala is catalyzed by D-alanine:D-alanine ligase (ADP) (6.3.2.4) (Neuhaus, 1962a,b; Ito and Strominger, 1962b).

The amino acid sequence of the uridine nucleotide is determined by the specificity of these enzymes. There are a number of growth experiments with *Streptococcus faecalis* R which suggest that critical substitutions may occur in the pentapeptide when the bacteria are grown in the presence of certain analogs of D-alanine (Snell and Guirard, 1943; Snell *et al.*, 1955).

It is the purpose of this communication to report the specificity profile of the D-Ala-D-Ala adding enzyme (UDP-NAc-muramyl-L-Ala-D-Glu-L-Lys:D-Ala-D-Ala ligase [ADP]) from *S. faecalis* R with respect to the terminal dipeptide moiety and compare it with the

* From the Department of Chemistry, Northwestern University, Evanston, Ill. *Received September 17, 1964.* Supported in part by a grant (AI-04615) from the National Institute of Allergy and Infectious Diseases; by a Public Health Service training grant (5 TI GM-626); a grant (GM 10006) from the Division of General Medical Science; and by grants from the Graduate School Research Fund of Northwestern University. Preliminary reports have been presented (Neuhaus and Struve, 1964a,b).