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Waterfall Sequence for Intrinsic Blood Clotting

Abstract. *A simple waterfall sequence is proposed to explain the function of the various protein clotting factors during the formation of the fibrin clot. When clotting is initiated, each clotting factor except fibrinogen is converted to a form that has enzymatic activity. This activation occurs in a stepwise sequence with each newly formed enzyme reacting with its specific substrate, converting it to an active enzyme.*

A number of mechanisms have been proposed to explain the function of the various clotting factors in the formation of the fibrin clot (*1*). Of particular importance is the concept that the protein-clotting factors interact in pairs in which one factor behaves like an enzyme and the other like a substrate. Through this interaction, the various clotting factors are converted in succession to active enzymes which eventually bring about the conversion of prothrombin to thrombin. The enzyme thrombin then converts fibrinogen to fibrin.

The route by which thrombin forms in cell-poor plasma has been called the intrinsic pathway, since it does not require the participation of substances extrinsic to the blood. A mechanism (*2*) which is consistent with most of the current investigations on the intrinsic clotting system is shown in Fig. 1. Many of these reactions have been demonstrated with plasma obtained from patients with congenital deficiencies and with partially purified preparations of clotting factors. The evidence supporting the sequence outlined in this report varies in its completeness from step to step, and we do not wish to imply a dogmatic view.

Several aspects of this proposed waterfall mechanism shown in Fig. 1 should be emphasized: (i) Each protein clotting factor shown occurs in plasma in an inactive or precursor form. These inactive proteins are listed on the left side of the waterfall along with their assigned Roman numeral (*3*). (ii) When clotting is initiated, each clotting factor except fibrinogen is converted to a form possessing enzymatic activity. The activated forms are shown on the right-hand side of the waterfall. (iii) The activation of each clotting factor occurs in a stepwise sequence with each newly formed enzyme reacting with its specific substrate and converting it to an active enzyme. The precise mechanisms by which most of these activation reactions occur are unknown.

In the test tube, this series of reactions is initiated by contact with a surface such as glass. This reaction, which is the first recognized event in the intrinsic clotting pathway, involves the conversion of Hageman factor to activated Hageman factor (Fig. 1, first step) (*4*). Many substances such as kaolin, barium carbonate, supercel, celite, bentonite, asbestos, and silicic acid mimic the glass effect. Plasma collected in glassware lined with paraffin, vaseline, or silicone (*5*) does not clot and may stay in the fluid state for 24 to 48 hours or longer. When transferred to ordinary glass containers, this plasma will clot in a few minutes. The molecular events occurring during the surface activation of Hageman factor are unknown. It is evident, however, that some rearrangement of the protein molecule occurs, forming or unmasking an active catalytic site in the protein (*6*). The physiological mechanism for the activation of Hageman factor in vivo is not known, although it presumably involves some enzymatic system with unknown clotting factors.

Once Hageman factor becomes activated, it converts plasma thromboplastin antecedent (PTA) to an activated form (*7, 8*). In vitro this reaction is accelerated several fold by $1 \times 10^{-5}M$ long chain saturated fatty acids (*9*).

The next reaction in this sequence is the activation of Christmas factor by activated PTA (*8, 10, 11*). In this reaction, activated PTA participates as an enzyme converting its substrate, Christmas factor, to an activated form. The reaction has an absolute requirement for divalent metal ions. At $5 \times 10^{-3}M$, calcium ions are the most effective, although other ions such as strontium, cupric, and zinc are also active (*12*). The reaction has an optimum *p*H of 8.0 (*13*).

The activation of Christmas factor is blocked by a number of inhibitors. Agents such as citrate, oxalate, and ethylenediaminetetraacetate are effective inhibitors by virtue of their calcium binding capacity. Another important inhibitor of this reaction is heparin (*11*) which apparently forms an inactive complex with activated PTA. This inhibitory effect is completely abolished by protamine sulfate or hexadimethrine bromide (*12*). Although heparin also interferes with the activation of antihemophilic factor (AHF) (*14*) and the formation of the thrombin fibrinogen complex (*15*), it is probable that a major site of action of heparin as a physiological anticoagulant is the blocking of Christmas factor activation. The war gas diisopropylphosphofluoridate (DFP) is also an inhibitor of this step (*11*) and reacts with activated PTA to form an inactive diisopropylphosphoprotein compound. The DFP-binding site in activated PTA (*13*) is the same as that in thrombin (*16*), that is, the hydroxyl group of serine in the peptide glycylaspartyl-seryl-glycine. The action of activated PTA on Christmas factor is also inhibited by a protein fraction found in plasma and serum.

The chemical events which occur during the activation of Christmas factor are unknown. It has been suggested that activation may involve partial proteolysis of the Christmas factor molecule since partially purified preparations of activated PTA contain esterase activity (*13*).

Once the Christmas factor becomes activated, it interacts with AHF and Stuart factor in the presence of calcium ions. The final product of this interaction was called Product I by Bergsagel and Hougie (*17*) and probably corresponds to activated Stuart factor (*18*). Biggs and Bidwell (*19*) and Fisch and Duckert (*20*) have studied the kinetics of these reactions and the relationship to AHF concentration. Recently, the activation of AHF by activated Christmas factor has been studied in detail by Lundblad and Davie (*14*). In this reac-

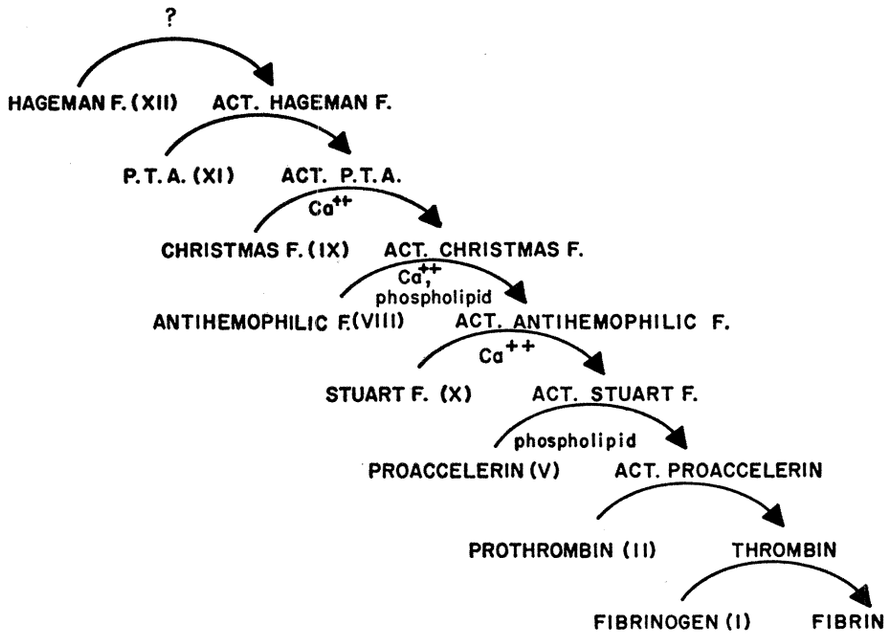

Fig. 1. Tentative mechanisms for the initiation of blood clotting in mammalian plasma in the intrinsic system. Abbreviations: F., factor; Act., activated; P.T.A., plasma thromboplastin antecedent. The term "Act. Proaccelerin" is probably a misnomer but was used in this figure instead of accelerin or prothrombin converting principle. Accelerin refers to a thrombin-modified form of proaccelerin; prothrombin converting principle, a term we have used elsewhere, does not identify the precursor of this enzyme. Hageman factor, Christmas factor, and Stuart factor are clotting factors named after the patients who were among the first observed in which the clotting deficiency was seen. This scheme does not represent all views held on the mechanism of blood coagulation (*32*).

tion, activated Christmas factor participates as an enzyme and converts its substrate, AHF, to an activated form. The reaction requires calcium ions and phospholipid. For the phospholipid requirement, a mixture containing equal amounts of phosphatidyl serine and phosphatidyl choline is most effective. The reaction is blocked by heparin. Once the AHF is activated, it in turn activates Stuart factor (*21, 22*). This reaction also has an absolute requirement for calcium ions (*21*).

The next event in this series of reactions is the interaction between activated Stuart factor and proaccelerin to form a prothrombin converting principle, activated proaccelerin (*23*). This reaction also requires the presence of phospholipid. No metal ion is required. Soybean trypsin inhibitor is a potent inhibitor of this reaction, presumably combining with activated Stuart factor.

Once proaccelerin becomes activated, it converts prothrombin to thrombin (*23*). Thrombin then converts fibrinogen to fibrin by partial proteolysis (*24*) which liberates two specific peptides from the *N*-terminal end of fibrinogen (*25*). In the presence of calcium ions and activated fibrin stabilizing factor (*26*), a carbohydrate component (*27*) and ammonia (*28*) are released, *N*-terminal glycine residues disappear (*29*), and a tough insoluble fibrin clot is formed. Recently, it has been proposed (*28, 30*) that the last reaction is a transamidation reaction which involves the β-carboxyl of asparagine of one fibrin monomer with the *N*-terminal glycine residues in an adjacent fibrin monomer. During this reaction, the carbohydrate component originally attached to an asparagine residue is liberated and immediately decomposes to sugar and ammonia.

The intrinisic system shown in Fig. 1 probably overlaps with the extrinsic system at the level of Stuart factor. In the extrinsic system, tissue thromboplastin and factor VII probably are responsible for the activation of Stuart factor and, consequently, AHF, Christmas factor, PTA, and Hageman factor are bypassed.

The scheme shown in Fig. 1 provides a simple and straightforward sequence for the interaction of the various clotting factors, their relationship to metal ions, lipids, and inhibitors. Omitted from the figure are the natural inhibitors of several of the steps. That the various activated clotting factors shown in Fig. 1 are enzymes is based upon simple enzyme kinetics with specific clotting substrates. Thus far, these studies have included all the activated clotting enzymes except activated proaccelerin. Doubtless, this sequence will require modification as new clotting factors are discovered, and as further studies may reveal the complexity of substances now assumed to be free of other clotting agents. After this manuscript was submitted, a strikingly similar coagulation scheme termed an "enzyme cascade" was reported (*31*).

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Phenethyl Alcohol Synergism with Mitomycin C, Porfiromycin, and Streptonigrin

Abstract. *Cyanide and phenethyl alcohol greatly enhance the lethal action of mitomycin C, porfiromycin, and streptonigrin on an exponentially growing culture of* Escherichia coli. *Dinitrophenol similarly enhances the lethal action of mitomycin C and porfiromycin, but only slightly that of streptonigrin. Phenethyl alcohol may be functioning in these experiments as an inhibitor of electron transport.*

The antitumor antibiotics mitomycin C and streptonigrin are similar in that both cause disruption of DNA metabolism (*1*). Mitomycin C, the more thoroughly studied of the two, causes cross-linking of the complementary strands of DNA (*2*). Both antibiotics have an amino quinone ring (*3*), and in the case of mitomycin C it appears that this ring must be reduced by the cell in order to make the molecule biologically active (*2*). We report here the synergistic effects of sodium cyanide, β-phenethyl alcohol, and 2,4-dinitrophenol on the bactericidal action of both antibiotics (*4*).

At a mitomycin C concentration of 4 μg/ml (Fig. 1), the curve showing the death rate of cells of *Escherichia coli* strain 15 Phe⁻ is biphasic, possibly because there are two populations of cells, mononucleate and binucleate (or double) cells, which are killed at different rates. Simultaneous addition of 0.01*M* cyanide with the antibiotic very rapidly kills all cells. Phenethyl alcohol (0.02*M*) and dinitrophenol (0.002*M*) are also potent synergists; sodium azide (0.01*M*) is less effective. In the absence of mitomycin C none of the synergists decreases viability (not shown). We have observed similar effects with *E. coli* strains B and W. The antibiotic porfiromycin, which differs structurally from mitomycin C only in that its aziridine nitrogen is methylated (*3*), shows the same pattern of effects when used at a concentration of 30 μg/ml.

Streptonigrin at a concentration of 10 μg/ml also gives a biphasic killing curve (Fig. 2). Cyanide and phenethyl alcohol synergize as with mitomycin, but dinitrophenol has only a slight effect (curve *B*). When phenethyl alcohol or cyanide is added to a culture which has been inhibited for 30 minutes with streptonigrin plus dinitrophenol, the cells again die very rapidly (curves *C* and *D*), showing that this antibiotic has not lost its potency and that the dinitrophenol does not antagonize the action of cyanide or phenethyl alcohol.

Because phenethyl alcohol is reported to be a specific inhibitor of DNA synthesis (*6*), this action might be the basis of its synergistic activity. If so, then cyanide and dinitrophenol might also exert their effects by stopping DNA synthesis. However, this interpretation is not correct, as is demonstrated by the following two experiments. (i) The *E. coli* strain 15 Phe⁻ was grown exponentially as described in Fig. 1. The cells were chilled, harvested, washed, and suspended in the same medium without phenylalanine for 90 minutes. Under these conditions the cells complete one round of DNA synthesis but are unable to start another (*7*), and so DNA synthesis ceases. In our culture, analysis by the diphenylamine test (*8*) showed that DNA content did not change after the first 60 minutes. After the 90-minute incubation, during which the viable cell count rose 50 percent to 10^8 cells per milliliter, mitomycin C was added, with and without phenethyl alcohol. With antibiotics alone the cells died more slowly than they did in an exponential culture (8×10^7 survivors after 15 minutes), while with phenethyl alcohol also present a potent synergism was again observed (5×10^4 survivors after 15 minutes). (ii) The thymine-requiring strain *E. coli* 15 T⁻Phe⁻ (*9*) was grown exponentially in the medium of Fig. 1, but with thymine present (4 μg/ml). This culture was harvested, washed, and resuspended at a concentration of 10^8 cells per milliliter in the same medium without thymine. Under these conditions no DNA was synthesized by the cells, and the cell count was constant for nearly an hour before "thymineless death" began. If mitomycin C at 8 μg/ml was added to the culture shortly after resuspension, 5×10^7 survivors remained after 5 minutes; but,

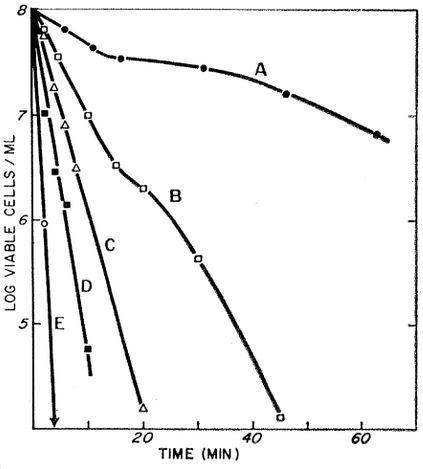

Fig. 1. A phenylalanine-requiring mutant of *E. coli* strain 15 was grown exponentially with aeration at 37°C in a tris-buffered minimal medium (*5*) containing glucose and phenylalanine, the generation time being 50 minutes. At 0 minutes there were 10^8 viable cells per milliliter, and mitomycin C (4 μg/ml) was added to all cultures. The following inhibitors were also added at 0 minutes: *A*, none; *B*, sodium azide (0.01*M*); *C*, dinitrophenol (0.002*M*); *D*, phenethyl alcohol (0.02*M*); *E*, sodium cyanide (0.01*M*).

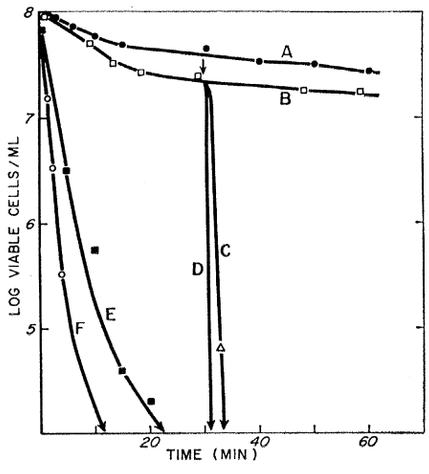

Fig. 2. Cells growing as in Fig. 1 were inhibited at 0 minutes with streptonigrin at 10 μg/ml. The following inhibitors were also added at this time: *A*, none; *B*, *C*, and *D*, dinitrophenol (0.002*M*); *E*, phenethyl alcohol (0.02*M*); *F*, sodium cyanide (0.01*M*). At 30 minutes (arrow) phenethyl alcohol (0.02*M*) was added to *C*; sodium cyanide (0.01*M*), to *D*.