BLOOD COAGULATION

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INTRODUCTION

This section covers the means, as far as we now know them, by which components of the blood plasma produce an insoluble protein meshwork, or gel, at a site of blood-vessel damage.

The plasma is normally completely fluid, containing all its proteins in soluble form. When the clotting system is activated at a site of blood-vessel damage, a series of proteolytic reactions is set going that ultimately results in the conversion of fibrinogen, which is soluble, to fibrin, which is not. Most proteins involved in the generation of the clot are plasma, not cellular, proteins. The total protein concentration in normal plasma is of the order of 7% (7 g/dl, or 70 mg/ml). The proteins devoted to clot formation account for less than 3 mg/ml, and of this the bulk is fibrinogen. The remaining plasma clotting proteins are present at much lower levels, ranging from prothrombin, at about 120 μg/ml (≈ 1.5 μM), down to factors VII and VIII at less than 0.5 μg/ml (< 10 nM).

Fibrin formation is just one part of the hemostatic system. The other components are the platelets, and the system by which damaged vessels contract under sympathetic nervous control. To some extent the platelets can function without the clotting system and *vice versa*, but the platelets require products of the clotting system to aggregate properly, and the clotting system requires platelets to form fibrin properly. These two parts of the system are therefore inextricably linked *in vivo*.

PLASMA and SERUM

Plasma is the fluid part of the blood, with all its clotting mechanisms intact and ready to go. **Serum** is clotted plasma. Usually serum is obtained by allowing whole blood to clot in glass (see *Contact Activation*), and then removing all the cells, and the clot, by centrifugation. Several of the clotting proteins are absent in serum, having been totally consumed in clot formation; the remainder are reduced to variable extents, and in some cases inactivated. Because plasma is rather unstable stuff, many laboratory procedures (chemistry, immunology, etc.) are done on serum. Clotting tests, however, are done on plasma. Do not confuse the two terms.

NOMENCLATURE

The nomenclature of the proteins involved in clotting is complicated and arbitrary, and there is almost no logic to it. The common names—the ones we will use—are in the left column of the table, accompanied by two columns of alternatives. You do not need to know the latter: they are included in case you do some reading elsewhere and come across them.

The cast of proteins in the table is arranged approximately in order of appearance, from initiation to final shutdown. The majority of clotting proteins are precursors of proteolytic enzymes, known as zymogens. The second major group is the cofactor proteins, which accelerate reactions. The plasma cofactors are high-molecular-weight (HMW) kininogen, factor V, and factor VIII. Two membrane-protein cofactors are critical: *tissue factor* is the initiator of coagulation, and *thrombomodulin* is central in switching off the clotting process. The remaining plasma protein, factor XIII, is also the precursor of an enzyme, but the enzyme is a transglutaminase, not a protease. It is involved in cross-linking fibrin strands.

Names, functions, and locations of blood coagulation proteins			
Common Name	**Common Alternative**	**Infrequent/Archaic**	**Function (location)**
Tissue factor	Thromboplastin	CD142, Factor III	Initiator; cofactor for factor VIIa in factor IX and factor X activation (subendothelium)
Factor XII	Hageman factor		Protease zymogen (plasma)
Factor XI		Plasma thromboplastin antecedent (PTA)	Protease zymogen (plasma)
Factor X*		Stuart factor	Protease zymogen (plasma)
Factor IX*		Christmas factor	Protease zymogen (plasma)
Factor VIII	Antihemophilic factor		Cofactor for factor IXa in factor X activation (plasma)
Factor VII*		Proaccelerin	Protease zymogen (plasma)
Factor V		Labile factor	Cofactor for factor Xa in prothrombin activation (platelets, plasma)
Prothrombin*	Factor II		Protease zymogen (plasma)
Fibrinogen		Factor I	Fibrin precursor (plasma, platelets)
Factor XIII		Fibrin-stabilizing factor	Zymogen of transglutaminase (platelets, plasma)
Thrombomodulin			Cofactor for thrombin in protein C activation (endothelial surface)
Protein C*			Protease zymogen (plasma)
Protein S*			Cofactor for activated protein C in inactivation of factors Va and VIIIa (plasma)
Antithrombin III	Antithrombin	Heparin cofactor	Protease inhibitor (plasma)
Tissue factor pathway inhibitor (TFPI)		Extrinsic pathway inhibitor (EPI); lipoprotein-associated coagulation inhibitor (LACI)	Protease inhibitor (platelets, plasma, endothelial surface)

* Vitamin K-dependent proteins

ZYMOGENS, PROTEASES

THE CASCADE. The clotting system consists of a series of proteolytic reactions, in each of which an inactive precursor (zymogen) of a proteolytic enzyme is converted to the active enzyme. These enzymes are called proteases or proteinases. Because each step in the series is enzyme-catalyzed, and one enzyme molecule can theoretically catalyze the formation of a very large number of molecules of product, a cascade has the capacity for enormous amplification (**Fig. 1**). For example, we may expect that one molecule of factor Xa, under ideal conditions, can generate about 1000 thrombin molecules per minute. If we have two such reactions in sequence, the *theoretical* amplification will be a million-fold per minute. *Et cetera*.

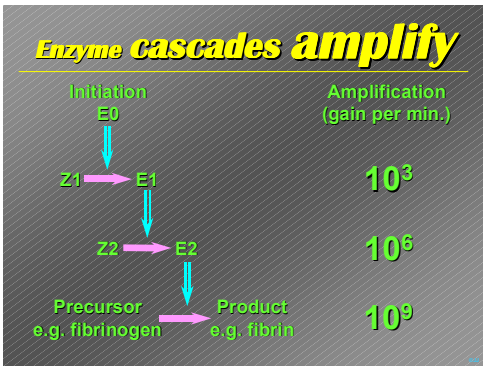

Figure 1

PROTEOLYSIS. For the conversion of a zymogen clotting factor to an enzyme, a lower-case "a" is added to the factor name. For example, the activation of the zymogen factor X produces the protease factor Xa. Similarly, one can (and we frequently do) write the activation of prothrombin to thrombin as the conversion of factor II to IIa. The activation of each zymogen is very similar, and comparable with the activation of the pancreatic zymogens chymotrypsinogen and trypsinogen. In each case a single specific cleavage in the precursor occurs (the activating cleavage) and a unique *serine* residue in the molecule becomes *catalytically active*, i.e. it is intimately involved in cleavage of the peptide bond. For this reason these enzymes (clotting enzymes, as well as chymotrypsin and trypsin) are called *serine proteases*.

SPECIFICITY. The clotting proteases exhibit great similarities in the catalytic part of the molecule (including the active serine). All of them (XIa, IXa, VIIa, Xa, and thrombin) are two-chain enzymes, and in each case the the chain with the catalytic apparatus—always formed from the carboxy-terminal piece of the zymogen—shows great homology with the pancreatic enzymes chymotrypsin and trypsin. In particular, they fall into the family of *trypsin-like* enzymes, because they cleave at basic amino acid residues. The clotting enzymes, however, differ in two respects. First, they have companion chains, whereas the pancreatic enzymes do not. Second, they have small inserts in the catalytic chain that have no counterpart in trypsin and chymotrypsin. These two additions together are largely responsible for their remarkable specificity.

Whereas trypsin, given the right conditions and enough time, will cleave most of the arginyl and lysyl bonds of a substrate protein, the clotting enzymes are much more specific. Activation of a clotting zymogen (e.g. the activation of prothrombin by factor Xa) involves at most two peptide-bond cleavages. In addition, unlike trypsin, which cleaves at both Arg and Lys bonds, clotting enzymes *only* cleave at Arg bonds. Overall, we have a general picture of the proteolytic mechanics of the clotting proteases being based on the pancreatic enzymes, with several additional features that enable them to play a much more specific role.

COFACTORS. Most clotting proteases require cofactor proteins to make the reactions that they catalyze go fast enough. Specifically,

- factor VIIa requires tissue factor,
- factor IXa requires factor VIIIa,
- factor Xa (acting on prothrombin) requires factor Va,
- thrombin activation of protein C requires thrombomodulin,
- activated protein C action on factor V and factor VIII requires protein S.

Cofactors have no enzymic activity themselves but they are, effectively, absolutely required. For example, although we can measure the activation of factor X by factor VIIa in the absence of tissue factor (in the laboratory), the reaction is about 20,000-fold faster in the presence of TF (Dr. Morrison's work, by the way). The other cofactors have similar effects.

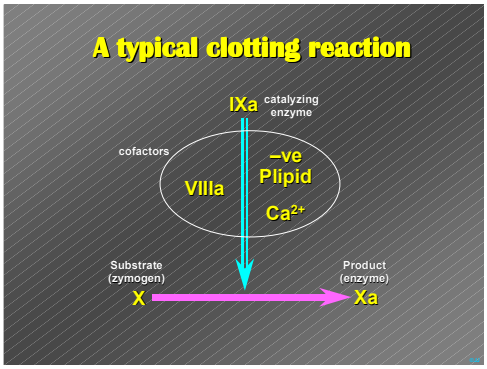

Figure 2

The standard depiction of a clotting, or other enzyme-catalyzed, reaction that I will use is shown in **Fig. 2**. In this example, the reaction being catalyzed is the conversion of factor X to factor Xa, shown by the solid purple arrow. The enzyme responsible is factor IXa, its proteolytic action on factor X being shown here by the open blue arrow. Any species shown beside the open enzyme arrow represent cofactors: in this reaction they are factor VIIIa, Ca^{2+} ions, and negatively-charged phospholipid.

INITIATION MECHANISMS

There are two initiators that you need to know about, but only one of them—tissue factor—is significant in normal hemostasis. The other is the contact system, which is the mechanism responsible for the clotting that occurs when blood or plasma comes into *contact* with "foreign" surfaces such as glass.

CONTACT ACTIVATION. Contact activation initiates a major laboratory test of the clotting system called the PTT (see *Clotting Tests*). We know that contact activation is not required for normal clotting because *people lacking any of the three proteins involved are hemostatically normal*. You must, however, at least know which the proteins are (**Fig. 3**). Contact activation generates factor XIIa in the presence of a "foreign surface", *factor XII, prekallikrein, and HMW-kininogen*, causing factor XI activation, which then activates factor IX and thus feeds into the "normal" clotting pathway (see Fig. 6). Although contact activation is not required for normal clotting, it may be sometimes involved in pathological situations that cause abnormal activation of the clotting system. In addition, contact activation is involved in inflammation through the ability of kallikrein to generate bradykinin from kininogen.

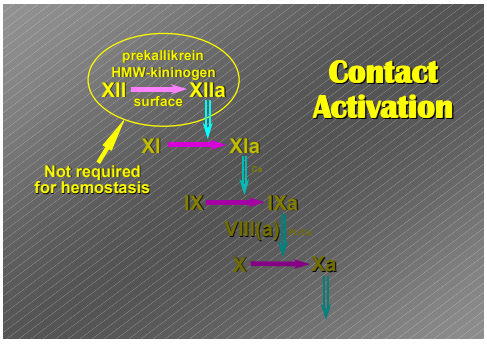

Figure 3

TISSUE FACTOR (TF). TF is the protein that initiates normal coagulation. Although a deficiency of TF has never been described,

TF "knockout" mice have been bred. Heterozygous mice, +/-, are born and are apparently normal, but homozygous mice, -/-, die in the embryo stage between 8½ and 10 days gestational age. This is the stage at which the embryonic vasculature is normally forming, but in these embryos what is usually seen is a small puddle of blood in the yolk sac. These data indicate that tissue factor is absolutely required for hemostasis.

TF is an integral membrane protein. It is normally expressed at only very low levels—if at all—in the endothelial cells, which line the blood vessel. Much richer in TF are cells that lie immediately behind the endothelium, chiefly the fibroblasts and smooth muscle cells. The TF level in cells is under transcriptional control, and can rapidly rise in response to several inflammatory and hormonal stimuli. Once the vessel wall is damaged, TF comes into contact with the proteins of the plasma. Because TF is a transmembrane protein it is unlikely to be released into the circulation unless there is massive tissue damage. The generally accepted idea is that it remains at the site. Recent reports suggest a major role also for the white cells, particularly monocytes and macrophages, in providing tissue factor in the initiation of hemostasis, and there is now definite evidence for tissue factor, in the form of microvesicles, circulating continuously. The source of this material is not yet completely clear, but there is no doubt that it is there; and it does raise serious questions about how the system copes with continous low-level stimulation. (See *Positive Feedbacks*, and their possible role in damping sub-threshold stimuli.)

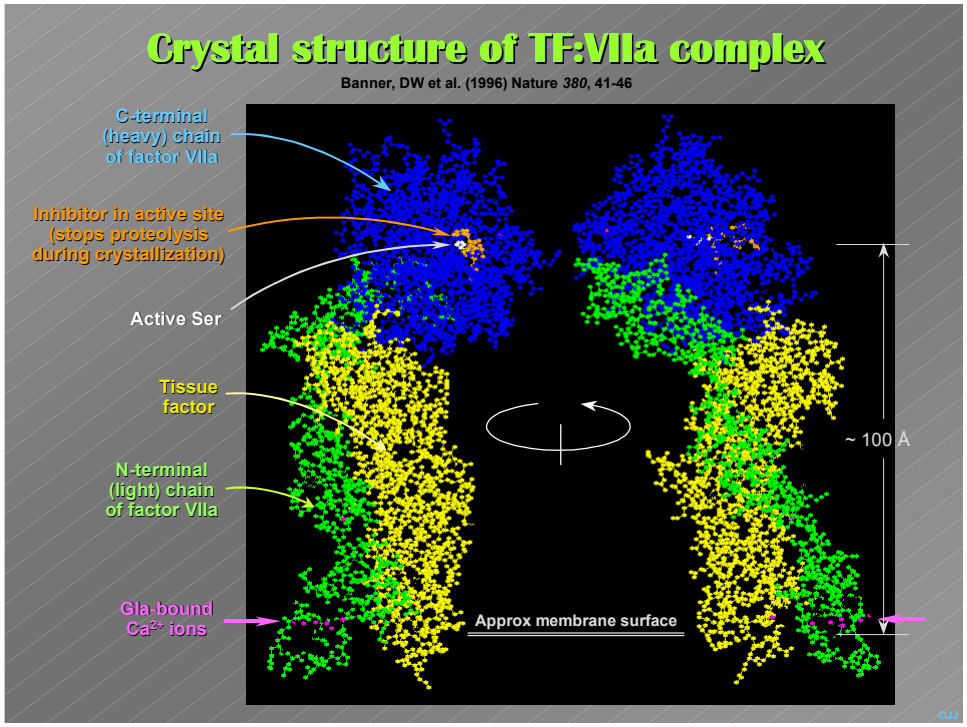

Figure 4

TF:FVII(a) COMPLEX. When TF comes into contact with the blood it forms a complex with factor VII, TF:VII, but this complex has no enzymic (proteolytic) activity. In order to gain activity, the factor VII part must be activated to form the proteolytic enzyme factor VIIa. This is done in a feedback reaction catalyzed by factor Xa (*Positive Feedbacks*). TF:VIIa (**Fig. 4**) is a proteolytically active complex that can activate *two* substrates–factor IX and factor X (**Fig. 5**). Note that the enzyme in this complex is factor VIIa, and TF is an essential cofactor. The activation of factor IX means that TF can initiate alternative routes of factor X activation: one direct, and the other via factors IX and VIII. Factor VIII is a cofactor. However, the active cofactor form, called factor VIIIa, does not exist in the plasma, and must be formed in a feedback. This is also described under *Positive Feedbacks*.

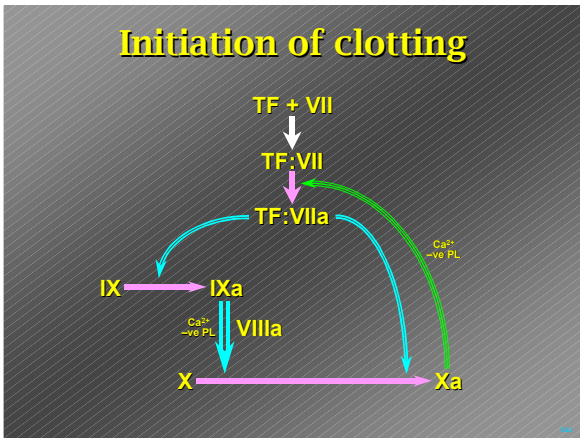

Figure 5

It is curious that there are two routes of factor X activation–one direct, and the other via factor IX and factor VIII. Although the teleological reason or the functional benefit of this is not proven, clinical facts and experimental studies tell us that the factor IX/VIII-dependent route is critical in the generation of factor Xa. Deficiency or defect in either factor VIII or factor IX causes hemophilia (A and B respectively).

PROTHROMBIN ACTIVATION

Having generated factor Xa, our next step is to activate prothrombin to form thrombin, and then convert fibrinogen to fibrin and cross-link it, completing the basic clotting pathways (**Fig. 6**). Prothrombin activation, by factors Xa+Va, is identical in form with the activation of factor X by factors IXa+VIIIa. Like factor VIII, factor V is a very large cofactor protein, and it is converted to its active cofactor form—factor Va—in a feedback reaction, by thrombin (see *Positive Feedbacks*). Both factor X and prothrombin activation also require negatively charged phospholipid and Ca^{2+}.

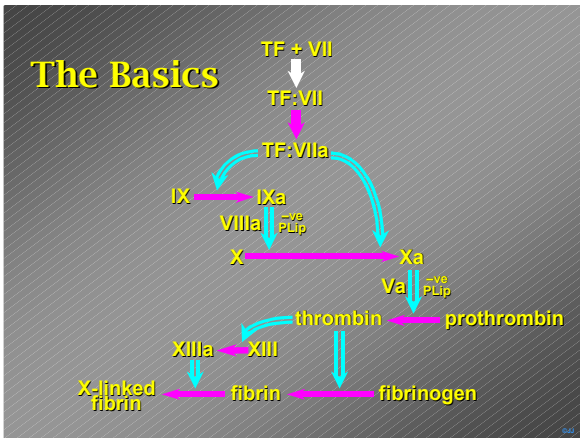

Figure 6

Although factor V is present in plasma, a significant proportion (about ¼ of the total) is present in the platelets. Upon platelet stimulation, this factor V appears at the platelet surface, and is concomitantly activated to the active form of the cofactor, factor Va. Activated platelets thus provide both the major cofactors for prothrombin activation: factor Va and negatively charged phospholipid (**Fig. 7**). Various evidence strongly suggests that it is the platelet's, rather than plasma's, factor V that plays the major role in prothrombin activation. The details of the interaction with negative phospholipid are described below (*Localization*).

Factor V is also where we find the most common hereditary risk factor for thrombosis, factor V Leiden. This variant, while it is converted normally by thrombin to the active-cofactor form, is defective in its ability to be inactivated by protein C (see *Negative Feedbacks*, below). The cause is the mutation of the Arg residue at the site where protein C cleaves and inactivates factor Va, to a Gln residue. The active cofactor is thus resistant to the action of protein C, and retains its activity, leading to abnormally high levels of thrombin generation.

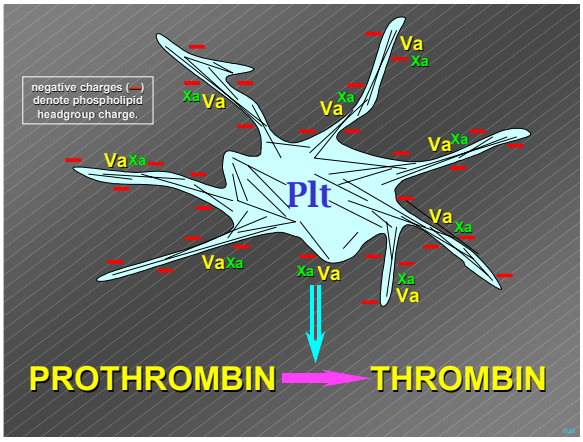

Figure 7

The protease product of prothrombin activation, thrombin, has numerous roles throughout hemostasis. It participates in both positive and negative feedback reactions; it activates platelets and factor XIII; and it is responsible for the generation of fibrin. In addition, many cell types have thrombin receptors, linking it to many other processes, including fibrinolysis.

FIBRIN FORMATION

Fibrinogen is a dimer of a trimer: it has two Aα chains, two Bβ chains, and two γ chains (**Fig. 8**). The major disulfide bonds are shown in the figure by darts (fibrinogen has a total of 29). The NH_2 termini of all six chains are close together in the *center* of the molecule. The COOH termini are in the globular region at the ends. The odd nomenclature (Aα, Bβ) reflects the fact that the Aα and Bβ chains are cleaved by thrombin, releasing the small A and B fibrinopeptides, and forming the α and β chains of *fibrin*. The γ chains are not cleaved. Both peptides carry a large negative charge for their size, and it is mainly the presence of these in fibrinogen that prevents it polymerizing.

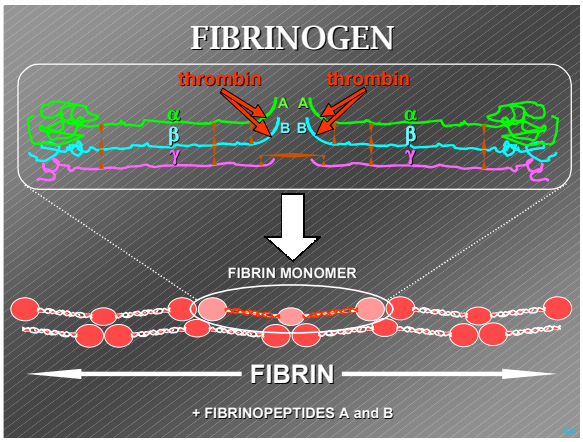

Figure 8

The product of thrombin action is a transient species called fibrin monomer. It consists of two α, two β, and two γ chains. Unlike fibrinogen, which is soluble, it polymerizes sponta-

neously, forming fibrin polymer. The initial polymer is characteristically two-stranded, with a half-staggered overlap. These protofibrils then go on to polymerize further, both longitudinally and laterally.

Polymerization initially occurs upon removal of the A peptide alone. Loss of the B peptide, which follows release of the A peptide, further stabilizes the polymer. Finally, the fibrin strands are cross-linked by a trans-glutaminase, factor XIIIa. This is formed from its zymogen, factor XIII, by the action of thrombin. Major cross-links are found mainly between the C-terminal regions of the γ chains, forming longitudinal cross links, and between the C-terminal regions of the α chains, forming both longitudinal and lateral cross-links. The cross-linking reaction itself involves the reaction of the ε-amino group of a Lys residue with the γ-amide of a glutamine residue, forming an "isopeptide" NH-CO bond between the two, with the loss of NH_3. The residues involved are quite specific: random cross-links between random Lys and Gln residues are not seen.

There are three additional things to note. (1) Because cleavage of the A peptide is all that is required for polymerization, *any test that measures clot formation is incapable of detecting defects either in cleavage of the B peptide or in cross-linking*. (2) Factor XIIIa is not just a fibrin cross-linker: it is capable of linking a variety of different proteins, including matrix and cell-surface proteins, and lipoproteins. These may be linked to each other, or may involve fibrin. Factor XIIIa is therefore a general anchoring enzyme as well as a fibrin cross-linker. (3) The Aα chains contain the binding site(s) for the platelet membrane protein GPIIb-IIIa (see *Platelets*).

LOCALIZATION

TISSUE FACTOR. Reactions that require tissue factor can only occur on a TF-bearing membrane (**Fig. 9** ➡). **Tissue factor does not exist in the platelets, and so the activations of factor IX and factor X by TF:VIIa cannot occur on platelet membranes**. However, the products—factor IXa and factor Xa—are not bound to TF, and can thus leave the TF-bearing membrane. They can then be localized by their interaction with negative phospholipid if it is available. They are not just *localized* there; they also *require* negative phospholipid for their activity on their substrates (factor X and prothrombin). Thus, if negative phospholipid is unavailable, they remain inactive.

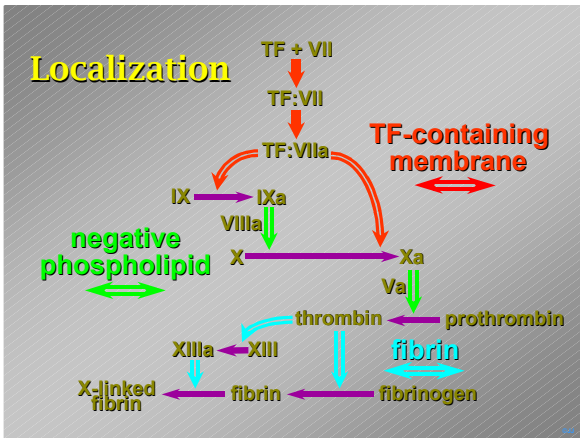

Figure 9

NEGATIVE PHOSPHOLIPID (**Fig. 9** ➡). Normal cells of all types have no significant negative (anionic) phospholipid head-groups in the outer membrane leaflet. When cells are stimulated (platelets, monocytes, etc.) or when they are damaged (sickle cells, hemolytic anemias, apoptosis, etc.), negative phospholipid—mainly phosphatidylserine (PS)—appears on the outer leaflet of the phospholipid bilayer. Negative phospholipid binds Ca^{2+} ions.

The mechanisms by which PS appears on the outer leaflet are getting a little clearer. In normal resting cells a phospholipid translocase is permanently active in ensuring that aminophospholipids (phosphatidylserine is the chief one) remain on the inside: any PS introduced to the outside is translocated back in. However, when the cytoplasmic Ca^{2+} level rises on cell activation, another protein becomes active, called (at least at present) a scramblase. This protein enables the rapid equilibration of phospholipid headgroups across the membrane, PS going out, and phosphatidylcholine (PC) going in. Stay tuned on this one...

VITAMIN K and γ-CARBOXYGLUTAMATE. The interaction of clotting proteins with negative phospholipid has to do with the post-translational carboxylation (in the ER of the hepatocytes) of glutamic acid residues in six *vitamin K-dependent proteins* (factors VII, IX, X, prothrombin, protein C, and protein S), and this carboxylation requires vitamin K (**Fig. 10**). The immediate NH_2-terminal regions of these proteins are very rich in the modified amino acid γ-carboxyglutamic acid (abbreviation Gla). Depending on the protein, the first 50 amino acids in the protein chain will contain

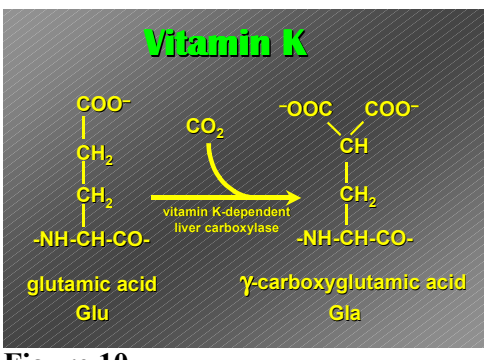

Figure 10

from 8-12 Gla residues, and this is called the Gla domain (see TF:VIIa structure, Fig. 4). From there on, no more Gla residues are found in these proteins—only ordinary Glu. γ-Carboxy-glutamate has a pair of carboxyl groups on the end of the aminoacid side chain, and this structure binds Ca^{2+}. It also happens that Gla residues often occur in pairs, and I show a pair in **Fig. 11**.

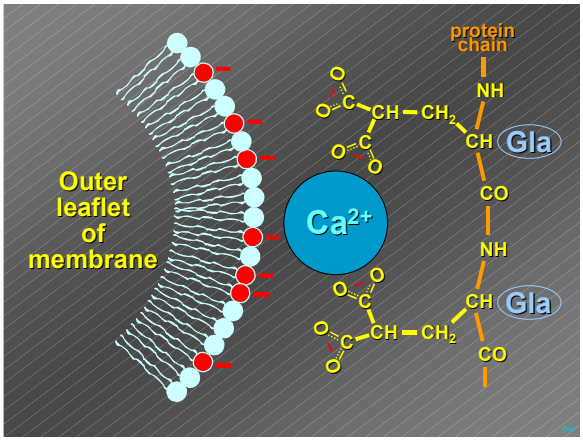

Figure 11

Things in reality are of course not quite as tidy as Fig. 11, and in practice a Ca^{2+} ion may be coordinated to three or four carboxyl oxygens, and sometimes just two. **Fig. 12** shows Ca^{2+} ions bound to the Gla residues of factor VII with the carboxyl oxygens shown in red. (The circle shows a particularly well presented Gla residue and the color scheme.)

In normal coagulation *in vivo* the major source of negatively charged phospholipid is undoubtedly the *activated* platelets. At the same time as the clotting pathway is initiated by the interaction of plasma factor VII with TF at the site of injury, so are the platelets stimulated at the site of injury—chiefly by collagen. This results first in adhesion, followed rapidly by inter-platelet aggregation and growth of a platelet plug. All this platelet stimulation leads to very large amounts of negatively charged phospholipid localized to the site of vessel damage. It may be noted in passing that, although factors VIII and V are not vitamin K-dependent proteins and have no Gla domains, they do contain hydrophobic domains that enable them to bind to membranes too.

VITAMIN K ANTAGONISTS: COUMARINS. Vitamin K antagonists are important anticoagulants that act by competing for vitamin K on the liver carboxylase that is responsible for putting the extra carboxyl groups on. The general name for the class of compounds is the coumarins, and Coumadin and Warfarin are common examples. The principal use for coumarins is in long-term anticoagulant therapy in patients who have suffered any one of a variety of thrombotic episodes. They have the great advantage over heparin that they can be taken orally, for years if necessary. Additionally—no small matter if a patient takes them daily for years—they are dirt cheap. Warfarin is also the active ingredient in rat and mouse poisons like Decon.

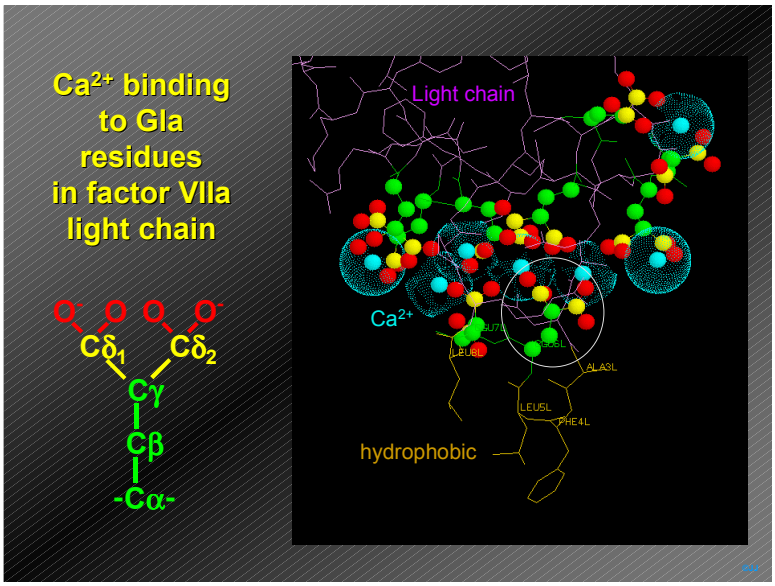

Figure 12

FIBRIN (**Fig. 9** ⇛). Although everything down to prothrombin binds to negative phospholipid, neither thrombin nor fibrin(ogen) does. The localizing surface for thrombin is the fibrin clot itself. Fibrin binds thrombin specifically though not very tightly ($K_d \approx 1$ μM), and has a very large capacity for the enzyme. Thus the great majority of thrombin binds to the fibrin that it is responsible for forming. However, because of the rather weak affinity, significant amounts of thrombin doubtless escape from the site of vessel damage. But, as you will see, there are potent controls to look after such leakage.

INHIBITORS

Localization of the clotting reactions to the site of vessel damage is the major mechanism by which the clotting system is controlled, but it is far from the only one. The next mechanism to consider is the inhibitors of the system. There are two major inhibitors, but they function in very different ways. Antithrombin III is one and tissue factor pathway inhibitor (TFPI) is the other (**Fig. 13**).

ANTITHROMBIN III. Antithrombin III (ATIII) is the major inhibitor of factor Xa and thrombin. **It is a very minor inhibitor of other enzymes**. It acts by forming a 1:1 enzyme:inhibitor complex with the target enzyme, blocking the active site. A partial deficiency of ATIII is a fairly common cause of thrombotic problems. Even in the concentration range of 40-60% normal, ATIII deficiency greatly increases the *risk* of thromboembolic problems like deep vein thrombosis and pulmonary emboli. Homozygous deficiency has never been described, which suggests that it may be fatal *in utero*. This is supported by the fact that homozygous deficiency in knockout mice is fatal to the embryo.

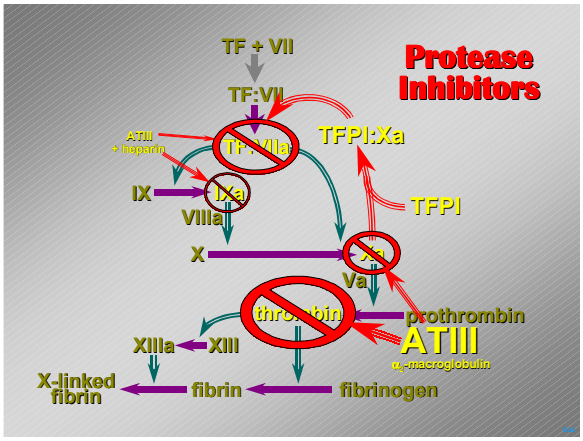

Figure 13

The concentration of ATIII in normal plasma, 4-5 μM, is more than 2-fold in excess over the total of all the clotting zymogens, so there is ample capacity in terms of the *amount* available. The reason that partial deficiency causes problems is probably not that the system runs out of inhibitor, but that the *rate of inhibition* is decreased in proportion to the concentration. The effect of rate on the overall curve that describes enzyme generation and inhibition is shown in **Fig. 14**. Note that the same total amount of enzyme was generated in each simulation here (100 nM), but the peak enzyme activity and the time over which the enzyme is active are controlled by the inhibition rate.

HEPARIN. Both in the clinical setting and *in vivo* the action of ATIII can be accelerated by heparin and similar materials. Heparin-like material is known to line the vessel wall, and is always present to maintain adequate inhibition capacity. Heparin accelerates the *rate* of action of ATIII on all its targets. At optimum heparin levels, the extent of acceleration is more than 100-fold. Thrombin and factor Xa remain the major targets: even though all the ATIII targets are inhibited faster, these two enzymes are still the by far the fastest inhibited. Unlike the coumarins (above), therapeutic heparin has to be injected or infused, and the halflife is short in the circulation; on the other hand—unlike the coumarins—the anticoagulant effect is immediate.

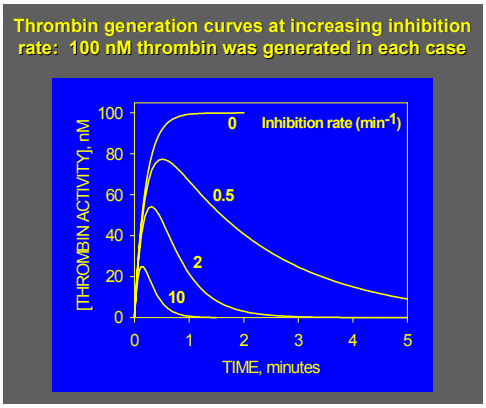

Figure 14

TFPI. Tissue factor pathway inhibitor is very unusual among inhibitors in that the active inhibitory species is formed in a *negative feedback* (**Fig. 15**). Specifically, TFPI reacts with factor Xa to form a TFPI:Xa complex, which *then* inhibits the TF:VIIa complex. TFPI's normal concentration in plasma is extremely low, around 1-2 nM (≈ 50 ng/ml). It can, however, be released by endothelial cells and platelets, and probably rises to much higher local concentrations under various conditions of cell stimulation. At present, because human deficiencies of TFPI have not (yet) been described, its significance is unclear, although studies of knockout mice suggest a major role in regulating the initiation of clotting.

POSITIVE FEEDBACKS

The clotting system is littered with positive feedback reactions. This term describes a reaction where a later enzyme catalyzes an earlier required reaction (**Fig. 15**). Thrombin and factor Xa are the enzymes responsible for the major feedback reactions in clotting (though there are others in the contact activation system), and they catalyze the activations of both zymogens and cofactors.

(1) One critical feedback in clotting is not usually considered as such; it is the activation of the platelets by thrombin. *Activated platelets* provide a negatively charged phospholipid surface for all the reactions of the vitamin K-dependent proteins; they provide factor Va in prothrombin activation (Fig. 7); and it is now known that they also potentiate another positive feedback, **(2)** the activation of factor XI by thrombin. The major "classic" feedbacks, also shown in the figure, are those of **(3)** thrombin on factor V and factor VIII, and of **(4)** factor Xa on TF:VII. Factor V and factor VIII could be called pre-cofactors, and they are converted to active cofactors (*not*

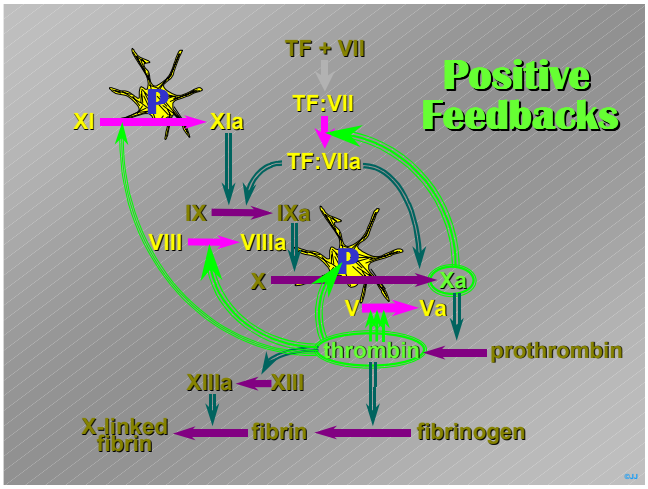

Figure 15

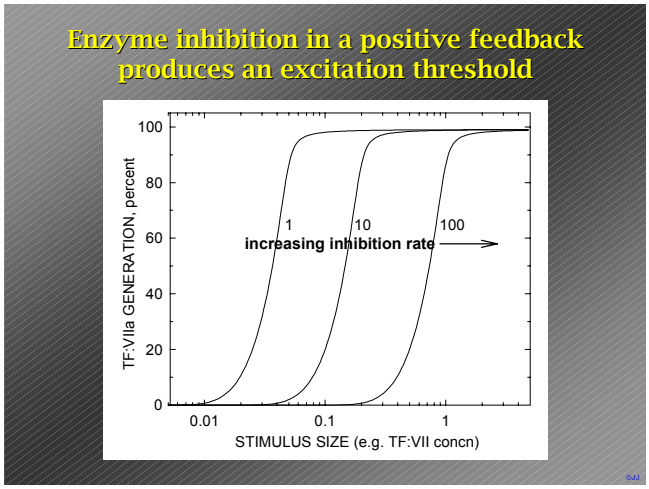

Figure 16

enzymes) on activation. Another feedback, not shown, is that of factor VIII by factor Xa; the reaction exists, but it may not be critical.

That clotting includes so many positive feedbacks suggests that they confer a major advantage in controlling the system. In this regard, note that factor Xa and thrombin are (1) by far the major targets of inhibitors, and (2) the only sources of feedback activations. It has been suggested (disclosure: by me) that this combination—potent inhibition of feedback enzymes—causes a significant means of system control: threshold behavior. Below the threshold level of a TF stimulus, feedback activation and stimulus amplification will not occur; whereas above the threshold they will (**Fig. 16**). This would mean that the clotting system is protected against very low levels of stimulus, to which it should not make a response in the form of a clot. In normal plasma, with all feedback targets in their inactive state, the clotting system is essentially idling very slowly. Only with a decent-sized stimulus do the feedbacks occur, and enable the system to get going.

NEGATIVE FEEDBACKS

Although they are not so numerous as the positive feedbacks, the clotting system includes equally critical negative feedbacks that shut the system down. They are distinguished from the straightforward action of inhibitors like ATIII by the fact that the feedback is initiated by a clotting enzyme—factor Xa or thrombin again—and therefore only occurs after system activation (**Fig. 17**).

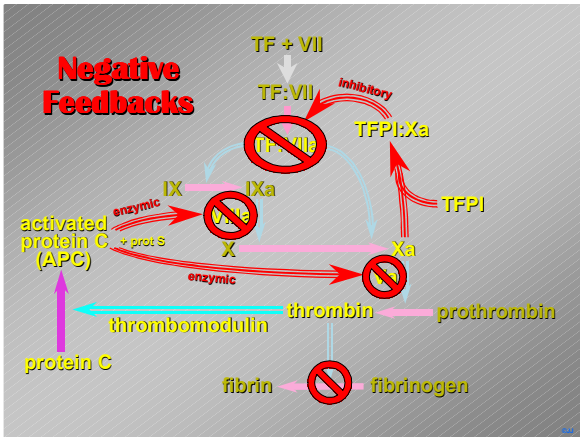

Figure 17

TFPI. Described above under *Inhibitors*, TFPI requires combination with factor Xa to form the inhibitory complex that inactivates TF:VIIa.

THROMBOMODULIN, PROTEIN C, PROTEIN S. The other negative feedback system is known to be critical in controlling the clotting response. Proteins C and S are plasma proteins, and a deficiency in either can cause massive thrombotic problems. Babies with homozygous deficiencies usually do not survive the first few days of life (they presumably survive *in utero* from sufficient of the mother's proteins passed across the placenta). Thrombomodulin, as mentioned above, is an endothelial-cell membrane protein that is *available to the flowing plasma throughout the vasculature.*

Thrombomodulin; protein C activation. Protein C, a zymogen, is activated to a protease, activated protein C or APC, by a thrombin:thrombomodulin complex. When thrombin binds to thrombomodulin, it not only becomes able to activate protein C; it also loses much of its activity on fibrinogen. In a very real sense, thrombomodulin changes thrombin from a *procoagulant* enzyme to an *anticoagulant* one. Notice, however, the different locations of the procoagulant and anticoagulant activities. Procoagulant thrombin is localized on the

fibrin clot itself, and will not be exposed to significant amounts of thrombomodulin. In contrast, any thrombin that leaks from the site of the clot will encounter thrombomodulin on the endothelium, and become anticoagulant.

Activated protein C. Once generated, this enzyme, in company with a cofactor, protein S, inactivates two major cofactors: activated factor VIII and activated factor V. Once again, the details suggest that it is activated cofactors that leak from the clot that are the major target: cofactors actively involved in clot formation are protected against inactivation. Note here too that APC is also involved in the fibrinolytic system, where is plays an additional anticoagulant, or pro-fibrinolytic, role (see *Fibrinolysis–Inhibition and Control*).

Inhibition. You may also note in passing that thrombin in complex with thrombomodulin is still subject to inhibition by ATIII. We thus get an overall picture of a system which, once thrombin escapes from a clot, ensures not only that it is inhibited, but also that it plays a positively anticoagulant role. All this is essential backup to the localization mechanisms.

DEFICIENCIES

While it is true that much of our knowledge of how coagulation works *in vivo* comes from clinical data on the bleeding disorders of patients with hereditary deficiencies of clotting factors, it must be remembered that these are very rare. For each one person with hemophilia (A or B), which is the most common hereditary bleeding defect, about 4-5000 other people will suffer a thrombotic episode during their lifetime. And we now know—recently, too—that hereditary abnormalities are in fact much more common in thrombotic than in bleeding disorders. For example, roughly 5% of the Caucasian population is heterozygous for factor V Leiden (see *Prothrombin Activation*). **Fig. 18** shows a *rough* table of the clinical severity of deficiency states. Deficiencies of factors VIII, IX, and XI are notable in that a patient can survive—though not well—a *total* absence of the factor. For example, before factor VIII replacement therapy for hemophilia A, even severely stricken patients with zero factor VIII would often live into their teens if not adulthood. In contrast, survivors of other severe deficiencies—factor VII and the proteins of the common pathway—are very much rarer and, as a rough rule, they are likely to have mutations that cause major but incomplete defects in protein *function* rather than a total absence of protein. Total deficiencies of proteins in the common pathway are probably usually fatal *in utero*.

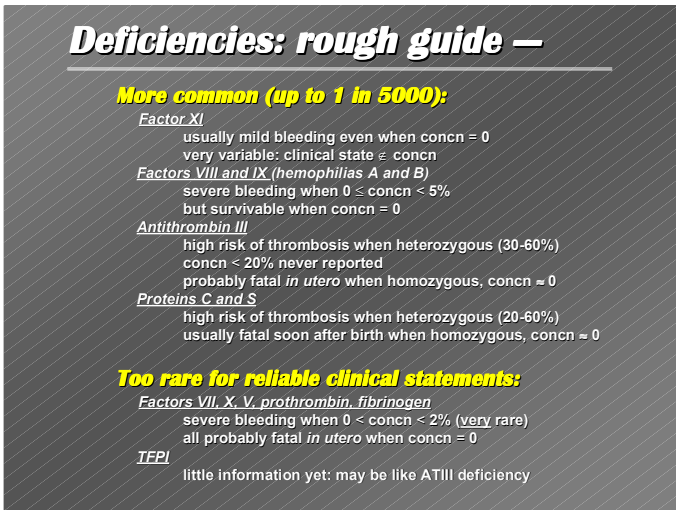

Figure 18

A speculative reason why hemophiliacs can survive a total lack of factor VIII or IX is that there is the alternative direct pathway for factor X activation by TF:VIIa, allowing at least a minimal hemostatic capacity to be maintained.

Factor XI deficiency merits mention. (It is largely confined to Ashkenazi Jews, and unlike the hemophilias the gene is autosomal and deficiency afflicts both sexes.) Unlike other deficiency states, where the clinical symptoms correlate quite well with the functional level of clotting factor, there is very poor correlation between the level of factor XI function and the severity of bleeding. Indeed some individuals with a total deficiency survive daily life with almost no bleeding episodes. Conversely, others may have a factor XI level as high as 20% normal, and have frequent, though mild, bleeding. Factor XI function in clotting is likely tied in with a role for both platelets and thrombin in activation of the protein, but the details are still a little murky (see *Positive Feedbacks*).

CLOTTING TESTS

There are two main screening tests of the clotting system: the prothrombin time (PT) and the partial thromboplastin time (PTT). (Occasionally the PTT is called the activated PTT, or aPTT: this is the same test.) Both the PT and PTT are clotting tests, i.e. they actually measure the time it takes to form a clot, but they use different means to initiate clotting. Both are done on "platelet-poor" plasma (i.e. < 5% normal platelet count), and

the patient's platelets are therefore irrelevant. Because activated platelets normally supply negative phospholipid for coagulation, another source of phospholipid must be supplied for these tests.

Also note that these tests are done on *citrated* plasma, i.e. the patient's blood was collected into a solution of sodium citrate as an anticoagulant (by convention, these are *blue-capped* collection tubes). Citrate is a chelator, which binds Ca^{2+} ions, and it thus blocks the Ca^{2+}-dependent reactions of clotting (i.e. most of them). In order to do a clotting test, sufficient Ca^{2+} must be added back to bring the Ca^{2+} concentration back to normal. It should also be noted that clotting tests cannot be done with plasma collected into EDTA. EDTA is a much more potent chelator of Ca^{2+} than citrate is, and the near-total removal of Ca^{2+} by EDTA causes rapid inactivation of plasma factor VIII, and to a certain extent factor V. It should be obvious that blood collected into heparin cannot be used in clotting tests either.

PT. Fig. 19 shows the reactions that are required to be normal to give a normal PT. The test is initiated with a crude preparation of rabbit tissue factor. Colloquially this is known as "brain", since it is made by acetone extraction of rabbit brain. More specifically, it is known as rabbit thromboplastin. Even though thromboplastin and tissue factor are technically synonymous, the word thromboplastin is often used to denote a crude preparation that contains tissue factor. The term tissue factor is reserved for the pure protein that interacts with factor VII to initiate coagulation. Since crude thromboplastin contains large amounts of negative phospholipid, other phospholipid does not need to be supplied in the PT. Apart from the plasma sample itself the only other requirement is Ca^{2+} (see above).

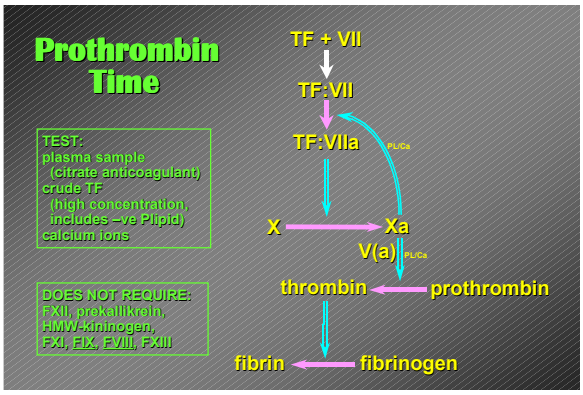

Figure 19

You must appreciate that the conditions of the PT are far removed from anything that can occur *in vivo*. The concentrations of tissue factor and phospholipid are extraordinarily high, and the direct activation of factor X by TF:VII(a) is therefore abnormally rapid. It is essential that you do not relate the conditions of the PT to the way in which clotting occurs *in vivo*: it is simply a laboratory test. In particular, it requires neither factor IX nor factor VIII, even though both are required *in vivo*.

The prothrombin time is also the standard test for monitoring coumarin therapy (see *Vitamin K Antagonists*). The PT does not tell anything about the level of the various required factors in the plasma, so monitoring is done on a purely empirical basis; specifically, the fractional lengthening of the clotting time. Commonly, coumarin (Warfarin or Coumadin) dosage is adjusted to give a PT of 1.5–2 × normal. Regular monitoring of the patient is essential.

PTT. The PTT involves activation of the contact system, which generates factor XIa (**Fig. 20**). This then activates factor IX, followed in turn by factor X, etc. The PTT does not involve tissue factor and thus does not require factor VII. The reactions that are required for a normal PTT are shown here. As I have emphasized, the contact system is not a part of normal hemostasis; and *contact-system proteins, although required for a normal PTT, are not required for normal clotting* in vivo. The proteins are factor XII, prekallikrein, and HMW-kininogen. The test itself requires an activating surface or compound. In addition, because the platelets have been removed, it requires a source of negative phospholipid; and, like the PT, it requires Ca^{2+}.

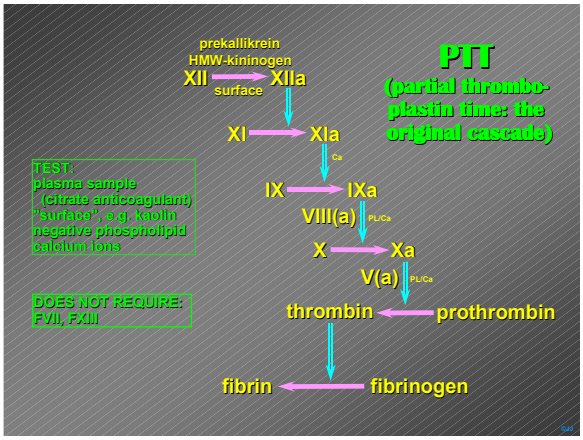

Figure 20

If heparin therapy is monitored the PTT is the test to use. However, heparin has a short half-life in the circulation, and in any case the risk of bleeding during heparin therapy is reasonably small; so in practice frequent monitoring is usually not required.

OTHER TESTS. A small number of other *general* tests are in fairly common use. They include:

Thrombin Time. This entails simply adding exogenous thrombin (not the patient's) to the patient's plasma and timing clot formation. It tests both fibrinogen cleavage and fibrin polymerization. Polymerization can be defective in the presence of *fibrin degradation products.*

Fibrin degradation products (FDPs). Serum made from normal plasma contains virtually no fibrinogen and no fibrin, which is insoluble. If FDPs are present in the patient's plasma (see *Fibrinolysis*), they will remain in the serum after clotting, and can be measured there. They are generally detected immunologically with antiserum against fibrinogen.

SPECIFIC FACTOR ASSAYS. If a patient has a long PTT and/or PT, more specific assays are done to determine the levels of the clotting factors that are suspect. For example, an abnormally long PTT, combined with a normal PT, would indicate a need for specific assay of at least factors VIII, IX, and XI. Conversely a defect in both the PT and PTT would indicate a need for specific assays of factor X, factor V, and prothrombin, which are required for both these screening tests. However, it should also be realized that multiple defects are not particularly unusual, and they may affect both the PT and PTT. One example is the broad lowering of clotting factor levels that is seen in severe liver disease.

PRODUCTS OF CLOTTING. Various by-products of clotting can be measured in plasma to confirm that the clotting system is running at rates above normal. This arises most commonly in disseminated intravascular coagulation and sometimes in thrombosis. By-products commonly used in this situation are platelet factor 4 and β-thromboglobulin, both of which are released from platelets on activation, and fibrinopeptide A, which of course is a by-product of fibrin formation. At present such tests are largely confirmatory: in particular, they cannot be used to predict even an immediate risk of thrombosis. Measurement of these various species is generally by radioimmunoassay or ELISA.

THROMBOTIC RISK. Patients at risk of thrombosis can be tested for the existence of factor V Leiden, which is a significant risk factor for thrombotic disease. In Caucasians the prevalence of the heterozygote is of the order of 5% of the population. Less common causes of thrombosis include heterozygous antithrombin III and protein C deficiencies, which can also be tested for. Still, however, for the majority of patients who suffer thrombotic episodes not related to atherosclerosis, an underlying specific cause cannot be identified.