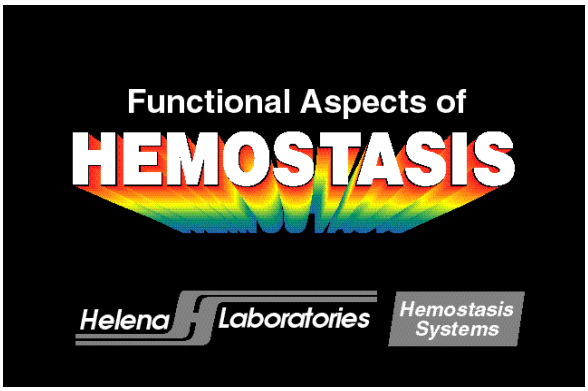
Functional Aspects of HEMOSTASIS

Cat. No. 5184

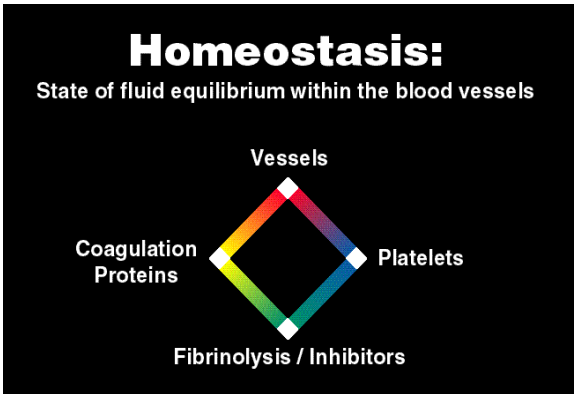
Helena Laboratories

Hemostasis Systems

Functional Aspects of Hemostasis

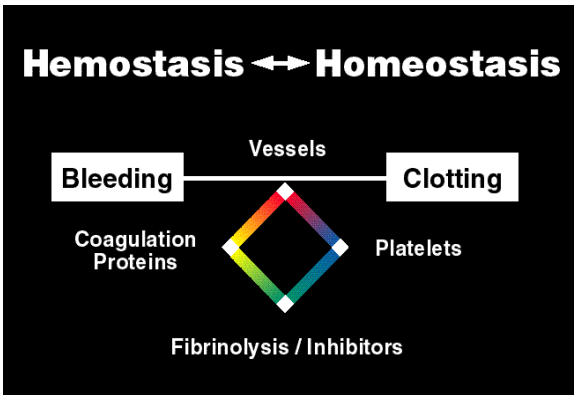


Slide 1
Our understanding of the complex interaction between the coagulation proteins, platelets, inhibitors, fibrinolytic system and blood vessels has grown significantly in recent years. As an educational service of Helena Laboratories, we're pleased to present a brief overview of this vital system as it relates to "Functional Aspects of Hemostasis".



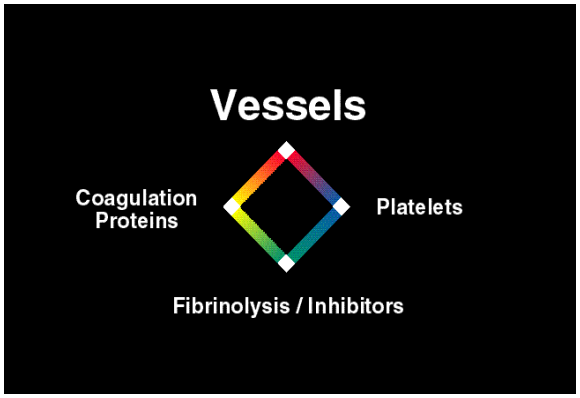
Slide 2
Homeostasis describes the normal condition of the circulatory system whereby fluid equilibrium is maintained within the blood vessels. Although most experts have expanded the term *hemostasis* ("arrest bleeding") to encompass what we are calling *homeostasis*, we will use the terms *homeostasis* and *hemostasis* in their more narrow context.

The components of homeostasis – the vascular system (blood vessels), platelets, the coagulation (plasma) proteins, and the fibrinolytic/inhibitor system – can be thought of as four points that form a perfectly balanced diamond. Deficiencies at any of the four points can cause bleeding or hemorrhage. In contrast, excess activation can result in potentially life-threatening thrombosis or coagulation.

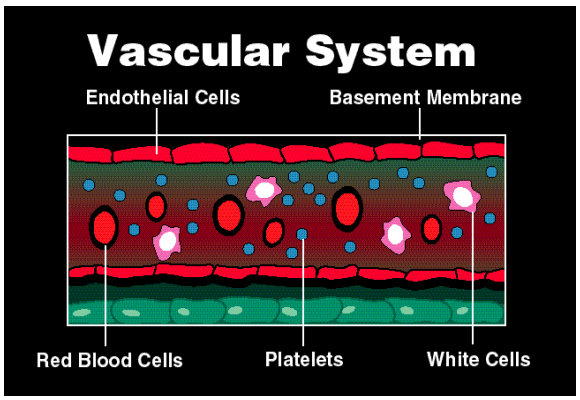


Slide 3
The complex process of homeostasis serves to maintain normal blood flow and provide an immediate and appropriate response to damage. Hemostasis occurs (and homeostasis is lost) when the system becomes unbalanced.

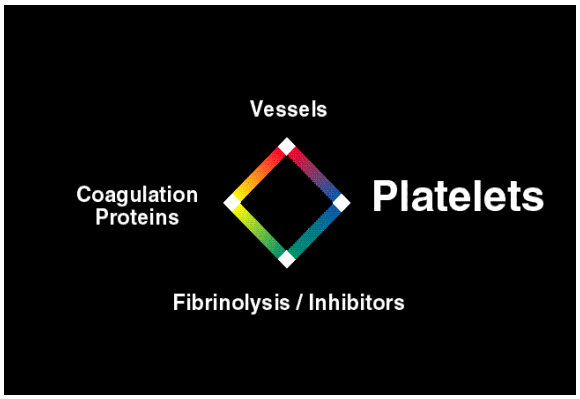
The body is in a constant state of subliminal vascular repair due to everyday wear and tear. The vascular response to injury coupled with platelet plug formation results in "primary hemostasis". The coagulation enzymes and the fibrinolytic/inhibitor system interact to localize and regulate the coagulation of blood. If equilibrium exists, blood loss from damaged vasular endothelial cells, or more severe forms of injury, is curtailed without jeopardizing the fluidity of the blood. Loss of dynamic equilibrium results either in significant hemorrhage or abnormal clotting (thrombosis).



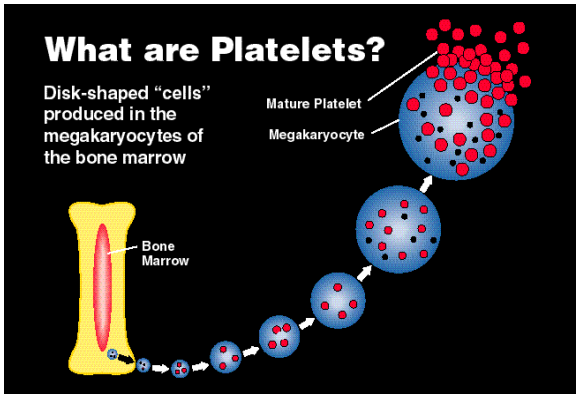
Slide 4
The first aspect of the diamond is the blood vessel itself. Of the component elements of hemostasis, the vasculature is least understood. In addition, we currently have inadequate laboratory tools to evaluate its functional characteristics. Although we will not discuss vascular defects or their causes, we will quickly review the structure of the vessel and its role in the arrest of bleeding.



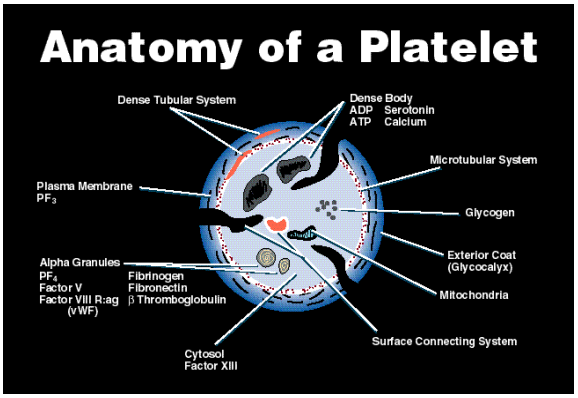
Slide 5
The fluid in the blood vessel contains red blood cells, white blood cells, platelets, and plasma proteins. The innermost endothelial cell lining is supported by the basement membrane and smooth muscle cells. Normally, the vessel wall binds neither platelets nor blood coagulation proteins. When the vessel is damaged, endothelial loss and exposure of the subendothelium to both platelets and blood coagulation factors initiates the process of platelet adhesion, platelet aggregation and blood coagulation.



Slide 6
The clinical manifestations of platelet and vascular defects are similar. Screening tests for these disorders are usually considered together. The diagnosis of a vascular defect is usually made after deficiencies in platelet number or function have been excluded. We'll spend a few minutes here reviewing platelets and their role in homeostasis and hemostasis.

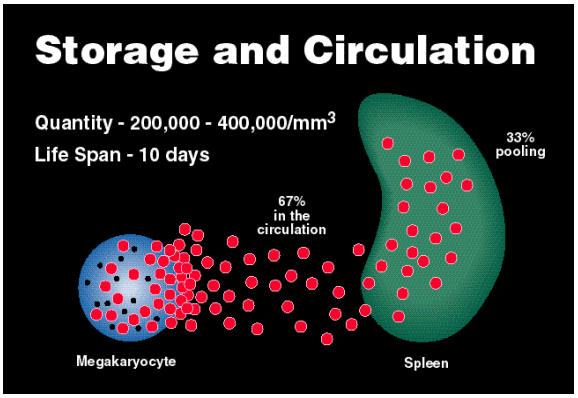


Slide 7
Platelets or thrombocytes are disc-shaped, anucleate "cell fragments" produced from megakaryocytes in the bone marrow. Megakaryocytes develop by a process of endomitosis or nuclear proliferation without cytoplasmic division. At the end of the eighth nuclear stage, cytoplasmic maturation begins and is characterized by the appearance of diffuse granulation and "demarcation membranes." These demarcation membranes represent formation of platelet plasma membranes. They eventually fuse, resulting in the "shedding" of cytoplasmic fragments of the megakaryocyte as platelets. The hormone thrombopoietin controls both megakaryocyte and platelet production.



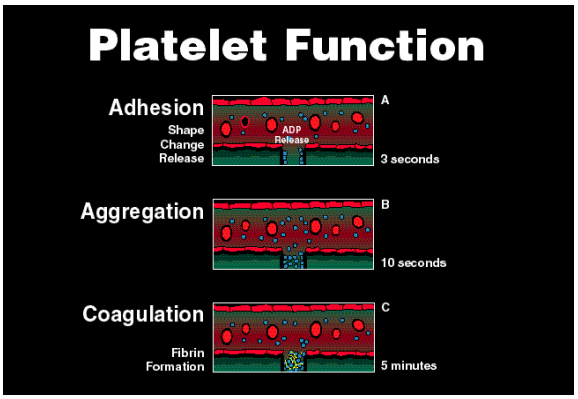
Slide 8

Here's a schematic representation of the structures within the platelet. Invaginations of the platelet membrane represent openings of the surface connecting system. This system is a series of channels which allows the platelet to take up substances from the plasma, as well as release intraplatelet substances from the storage granules. The majority of these storage granules are of the alpha type, containing platelet fibrinogen, fibronectin, Platelet Factor 4 and Factor VIII-related antigen (von Willebrand's factor). The second most common granule is the dense body, a storage site for non-metabolic ADP and ATP, serotonin, and calcium.



Slide 9

After release from the bone marrow, platelets circulate in the blood. In the normal individual, 67% of platelets are in the general circulation, with approximately 33% pooled in the spleen. Platelet concentration is normally 200,000 to 400,000 platelets per cubic millimeter of blood. Platelets may be counted by phase microscopy or by electronic particle counting equipment. Newer techniques and instruments now estimate platelet size. Platelet sizing is thought to be a good indicator of platelet production and/or platelet destruction. In general, the younger, robust platelet is larger. The normal life span of a platelet is 8 to 10 days.



Slide 10

The multifunctional role of platelets – adhesion, aggregation and interaction with the coagulation proteins – provides the primary defense in a challenge to the hemostatic system.

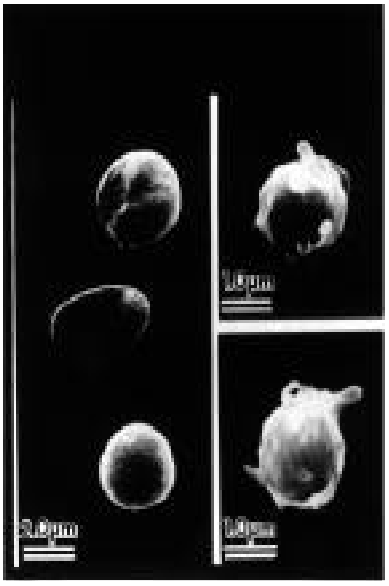
Functional *adhesion* is the first role of platelets. In vessel "A" we can see the early reaction to injury. The platelets adhere to the exposed collagen in the basement membrane of the blood vessel. Shape change and release of ADP occurs.

Next, *aggregation* begins. Aggregation is the sticking of platelets to other platelets to form a plug. Once the platelet adheres and aggregates, further reactants are released from the storage granules of the platelet. Additional platelets aggregate in response to the released ADP, undergo viscous metamorphosis and the "release reaction" from the storage granules stimulates aggregation of still other platelets. This cyclic stimulation results in the formation of a mass of aggregated platelets, the "primary hemostatic plug".

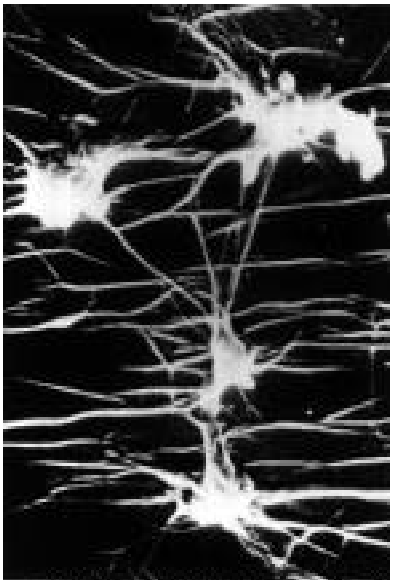
As platelets fulfill their roles in forming the primary hemostatic plug, there is interaction with the

plasma coagulation proteins. In block “C”, we see the end result of the *activation of the coagulation proteins*, the formation of fibrin strands in and around the platelet plug to form a stable clot to stop the loss of blood from the vessel. Finally, as the fibrin clot stabilizes, the platelet pseudopodia contract, tightening the clot.

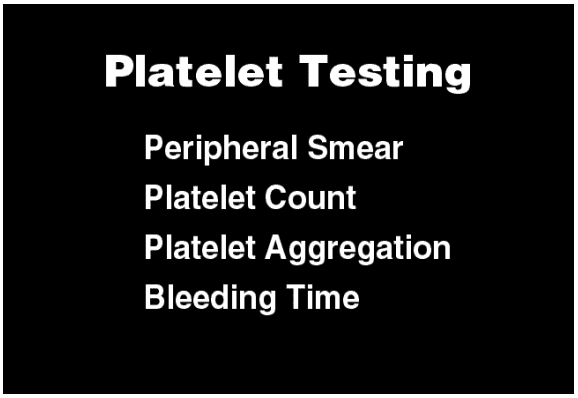
Not shown in this slide is an event that occurs much later, *platelet retraction*, which is the physical contraction of the platelet-fibrin plug.



Slide 11
Let’s take a look at real platelets. Pictured here are electron photomicrographs of platelets, kindly provided by the late Dr. Marion Barnhart of Wayne State University School of Medicine, Detroit, Michigan. On the left side of the slide, we see three relatively normal disc-shaped platelets. On the right side of the slide, we can see the beginnings of pseudopod formation of “activated” platelets.



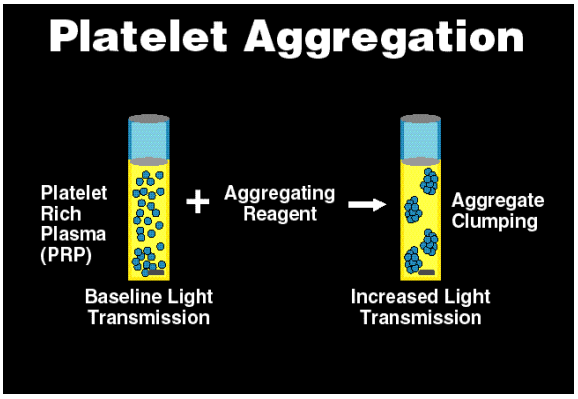
Slide 12
Here we can see more clearly how platelets adhere (to glass in this instance) and how they aggregate or stick to one another. Note how the elongated, tentacle-like pseudopods form a mesh that reduces blood flow to allow the subsequent formation of fibrin. This is particularly important in dealing with arterial hemorrhage.



Slide 13
Low numbers of circulating platelets (thrombocytopenia) and abnormal platelet function predispose patients to excessive bleeding. Platelet testing in the clinical laboratory should include both quantitative and qualitative tests:

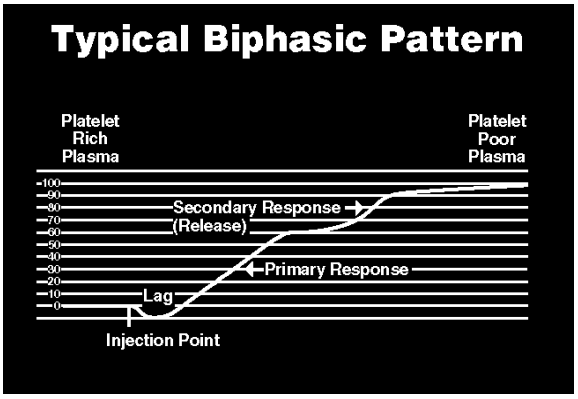
1. Peripheral smear – checks on quantitation as well as morphology.
2. Platelet count – accurately quantitates platelets.
3. Platelet aggregation – proper use of various agonists determines response to stimuli and demonstrates platelet function.
4. Bleeding time – measures platelet function (and to some extent vascular integrity), however, the lack of standardization and technique problems limit its usefulness.

Let's look in more depth at the tests used to assess platelet function.

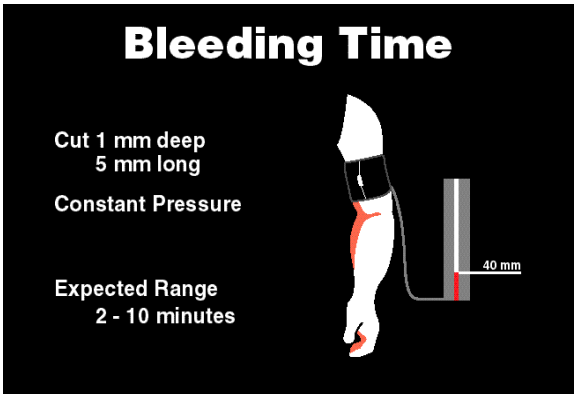


Slide 14
Platelet aggregometry studies are in-vitro assessments of platelet function. In principle, the test determines whether platelets will interact with other platelets to form platelet clumps when stimulated with various types of aggregating agents (agonists). Collagen, epinephrine, ADP, arachidonic acid, and ristocetin are commonly used.

The test begins by measuring the optical density of platelet-rich plasma (PRP). When an agonist is added to the platelet-rich plasma, the platelets clump, the plasma becomes less turbid and light transmission through the plasma increases.



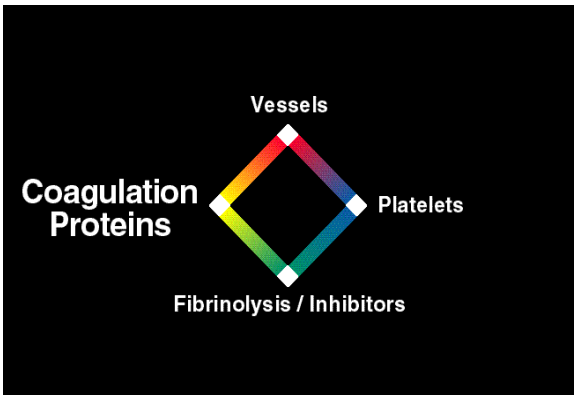
Slide 15
A typical aggregation curve using epinephrine is depicted in this slide. Beginning at the bottom of the graph, baseline is established by passing a light beam through platelet-rich plasma (PRP) and setting transmission at 0%. Epinephrine is added, the platelets begin to aggregate or form clumps, which allows more light to pass through the cuvette and the recorder shows an increase in light transmission. The direct aggregation of platelets by the aggregating agent is called "primary aggregation." With some agonists, such as epinephrine, this is followed by a second wave of aggregation. This secondary phase results from endogenous release of ADP from the platelet dense bodies (platelet release reaction), triggered by the aggregating agent.



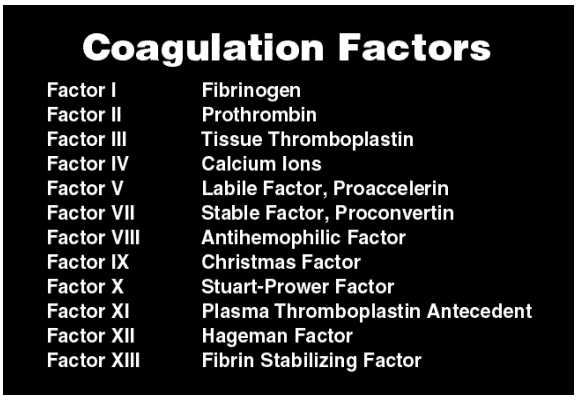
Slide 16
Bleeding Time or Template Bleeding Time is a basic screening test of platelet function. When first introduced, the Duke bleeding time method involved making a small cut in the patient's ear lobe and noting the time for the wound to cease bleeding. This test offered some measure of platelet function, but results were inconsistent due to wound size and variability in tissue from individual to individual.

For better standardization, Dr. Ivy proposed a forearm incision using a blood pressure cuff to apply a constant pressure. Dr. Mielke introduced a template to assure a consistent 1 mm deep x 5 mm long incision. This method, referred to as the Modified Ivy Bleeding Time, the Template Bleeding Time, or the Mielke Bleeding Time, is still currently in use. A bleeding time of 2 to 10 minutes is considered normal. Patients with prolonged bleeding times should be further evaluated by platelet aggregometry and other tests.

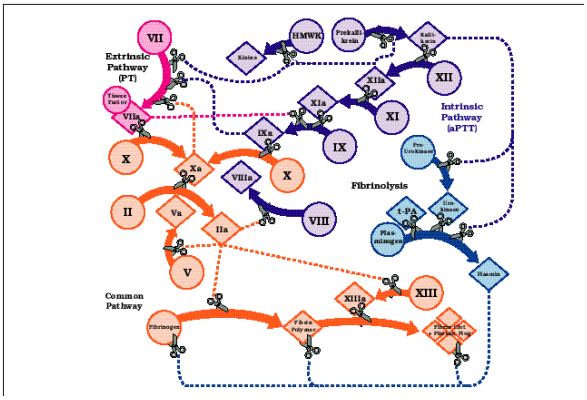
Patients with platelet counts of $\leq 100{,}000/mm^3$ need not be tested since the bleeding time will be prolonged even if platelet function is normal. Patients should not be tested if they have ingested aspirin-containing drugs during the previous 10 days days.



Slide 17
The next point on the "Homeostasis Diamond" we will discuss involves the coagulation proteins. The term "coagulation" has been loosely used to describe all the functions of hemostasis. For our purposes, we will use "coagulation" to define the plasma clotting proteins.



Slide 18
There are twelve principal coagulation factors. Early investigators often used their own "coined" nomenclature when referring to the same protein. Sufficient knowledge was eventually obtained about the biochemistry and physiology of these factors to allow experts to develop a more standardized nomenclature based on the use of Roman numerals. To date, twelve coagulation factors have been assigned the Roman numeral designations I through XIII. (A protein identified as Factor VI was later found to be a precursor to Factor V, so the Factor VI designation was dropped.) Several other proteins have been described that are uniquely different from Factors I through XIII, but as yet they have not been assigned a Roman numeral.

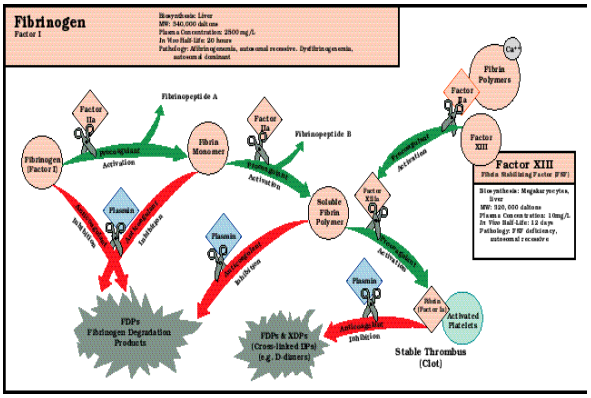


Coagulation Factors

Fibrinogen Group
Factors I, V, VIII and XIII

Prothrombin Group
Factors II, VII, IX and X

Contact Group
Factors XI, XII
Prekallikrein (Fletcher Factor)
High Molecular Weight Kininogen
(Fitzgerald Factor)



Slide 19
This representation of the "coagulation cascade" includes a graphic scheme of the flow once the clotting mechanism is initiated. These reaction sequences can also be represented as distinct pathways (extrinsic and intrinsic) which collectively lead to the formation of the prothrombinase complex. This complex generates thrombin, the enzyme responsible for the conversion of circulating fibrinogen to fibrin. It is this conversion of fibrinogen to fibrin that is measured in the laboratory by clotting assays.

Slide 20
It may be easier to understand the function of the various coagulation factors if we think of them in groups.

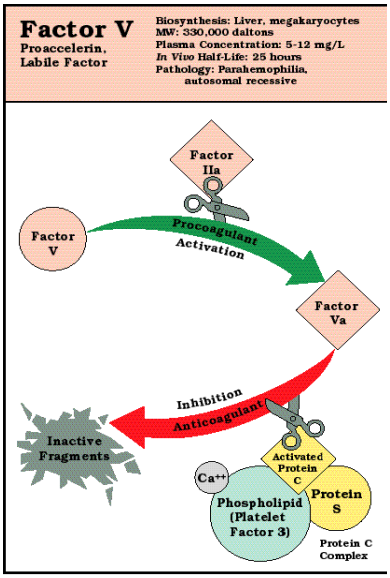
Factors I, V, VIII, and XIII, the *Fibrinogen Group,* are large proteins cleaved by serine proteases. These factors are excellent enzyme substrates and are heat labile.

Factors II, VII, IX, and X, the *Prothrombin Group*, are referred to as the Vitamin K-dependent factors. They require Vitamin K for synthesis of γ–carboxyglutamic acid responsible for binding ionized calcium; they are functionally suppressed by oral anticoagulants. These low molecular weight proteins are precursors of potent enzymes (serine proteases).

Factors XI and XII, prekallikrein, and high molecular weight kininogen, collectively referred to as the *Contact Group*, are intrinsic pathway initiators of the clotting mechanism. They trigger the coagulation cascade in response to various activators or stimuli.

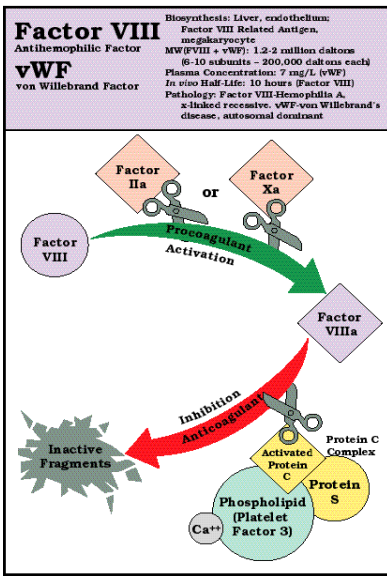
Slide 21
The next several slides have been reproduced from Helena's Hemostasis Pathways wall chart. Scissors indicate cleaving actions that activate precursor proteins (called zymogens) into fully functional clotting factors (designated by a small "a" meaning activated). Green arrows represent procoagulant reactions and red arrows represent anticoagulant reactions.

This slide depicts the formation of fibrin by the action of thrombin on circulating, soluble fibrinogen. Fibrin is an insoluble derivative that is further stabilized by a cross-linking reaction mediated by the activation of Factor XIII (shown in inset). Fibrin formation through the coagulation mechanism simultaneously results in the activation of the fibrinolytic system, which controls fibrin formation by its destruction or lysis.



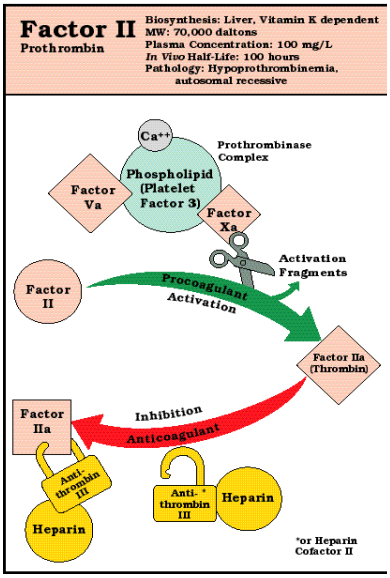
Slide 22

As in the previous slide for fibrinogen, we see inhibition of Factor Va as a proteolytic breakdown into non-functional protein fragments (depicted by the gray "explosions"). This kind of cleavage is caused here by the Protein C complex. Note that thrombin (IIa) is the activator here, just as it was in its more well-known role in converting fibrinogen to fibrin. In platelets, Factor V is stored in the alpha granules, and released to bind to the platelet membrane upon platelet activation.



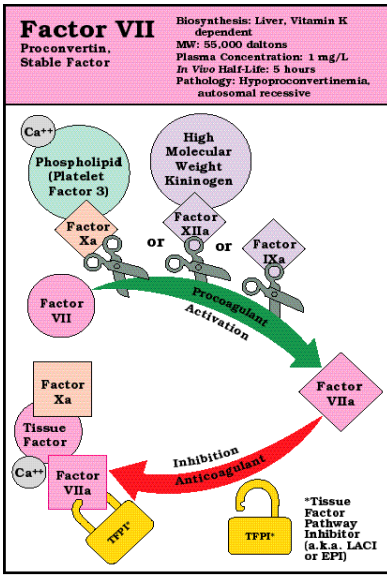
Slide 23

Factor VIII is the fourth member of the Fibrinogen Group of large coagulation proteins. It is well-known as the antihemophilic factor because its absence is the cause of classic Hemophilia A, as manifested in the descendants of Queen Victoria. Factor VIII is important for many reasons. Like fibrinogen, Factor VIII is an acute phase reactant whose level can rise to several times its normal amount in a variety of disease conditions. Factor VIII is bound to von Willebrand Factor, which interacts with platelets (as does fibrinogen), thus linking the plasma protein coagulation system with platelet response to vascular injury.

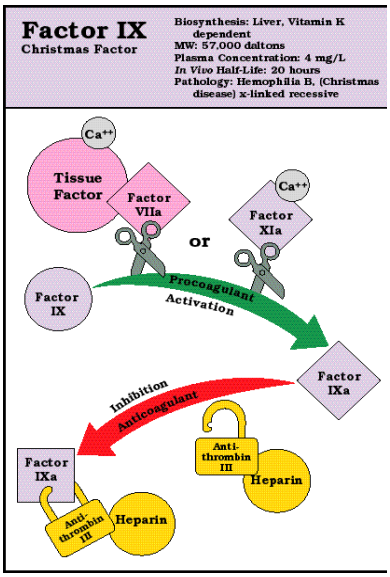


Slide 24

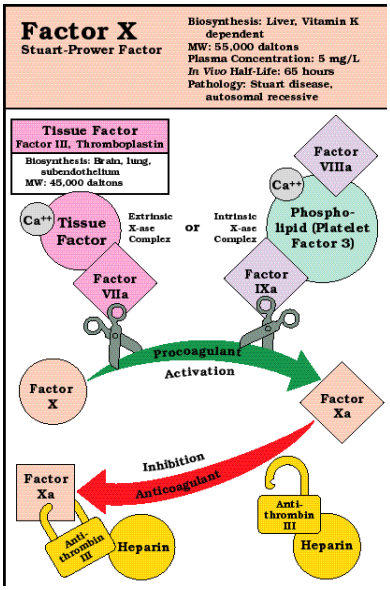
When prothrombin is activated by Factor Xa, a tremendously potent procoagulant enzyme (thrombin) is liberated. Once thrombin is formed, it can activate many of the other coagulant precursors, including auto-activation of prothrombin itself. The prothrombinase complex typifies many of the coagulation cascade reactions. While Factor Xa is the serine protease that actually cleaves prothrombin (therefore Factor Xa is "holding the scissors"), the other cofactors (Factor Va, Ca^{++} and phospholipid) make the reaction occur many thousands of times faster. Similarly, the inhibition of thrombin by antithrombin-III (shown as a padlock) is only effective in the presence of heparin-like substances.



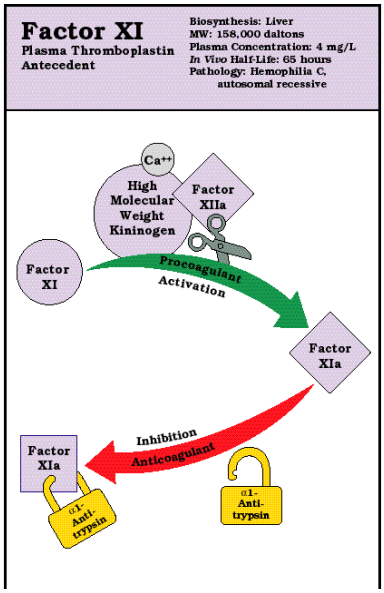
Slide 25
Factor VIIa, working in concert with Tissue Factor (thromboplastin), is currently considered by many experts to be the most important promoter of coagulation. As the only recognized physiological inhibitor of the extrinsic pathway, Tissue Factor Pathway Inhibitor is gaining acceptance as an extremely important regulator of hemostasis. (The name Tissue Factor Pathway Inhibitor (TFPI) was suggested in 1991 in place of EPI or LACI.)



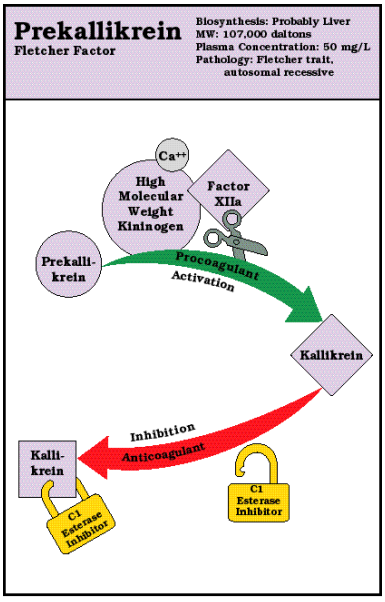
Slide 26
With the recent demonstration that Factor IX can be activated equally as well by Tissue Factor/Factor VIIa as it was known to be by Factor XIa, the distinctions between the extrinsic and intrinsic systems have begun to blur. The name Christmas Factor stems from Stephen Christmas, a young boy who was first described with Hemophilia B, or Christmas Disease.



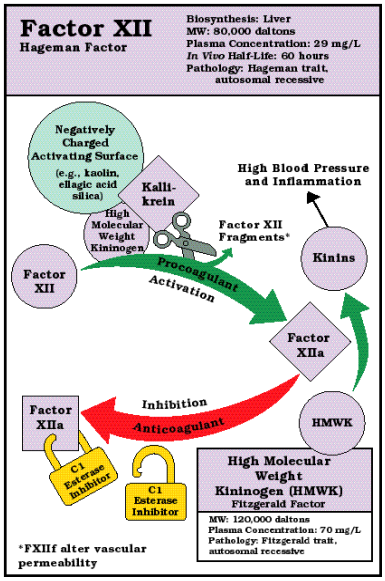
Slide 27
The conversion of Factor X to Xa can be accomplished by either an extrinsic or an intrinsic factor complex, and this step is the beginning of the common pathway. Like Factors IIa, VIIa, XIa, XIIa, kallikrein and plasmin, Factor Xa is inhibited by AT-III/Heparin. And, as compared to Factor IIa (thrombin), Xa is especially sensitive to low molecular weight heparin.



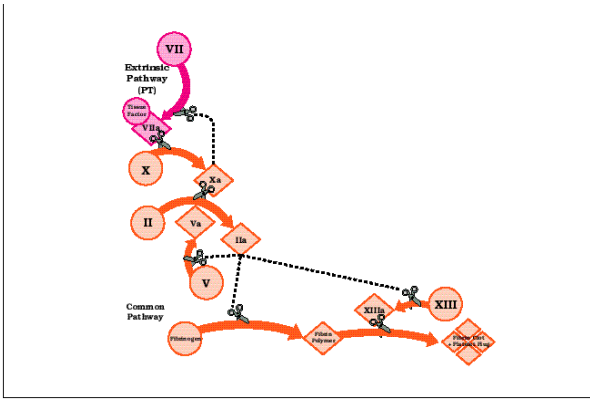
Slide 28
Factor XI was the first of the contact system factors (Factor XI, Factor XII, prekallikrein and high molecular weight kininogen) to be discovered, and its deficiency is most commonly seen in Ashkenazi Jews. Factor XIa is primarily inhibited by α_1-antitrypsin (a.k.a. α_1-protease inhibitor), an acute phase reactant whose deficiency often coincides with emphysema and liver disease.



Slide 29
A deficiency of prekallikrein (Fletcher Factor), another member of the Contact Factor Group, can cause a prolonged APTT, but does not appear to cause any hemostatic defect. Its more important roles are probably as an activator of fibrinolysis (i.e. plasmin) and in kinin production which involves vascular constriction and dilation.



Slide 30
Factor XII and high molecular weight kininogen (HMWK) are the remaining two contact factors. All four of the contact factors associate on negatively charged surfaces, such as glass (and other activators used in APTT reagents, e.g., kaolin or ellagic acid) and collectively activate the intrinsic system, the fibrinolytic system, the complement system and, via kinins and Factor XII activation fragments, the vascular system.



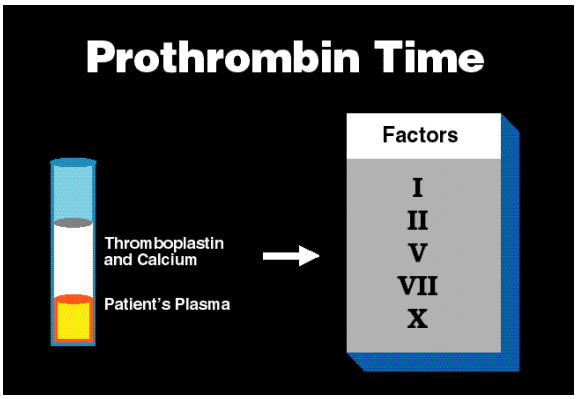
Slide 31

Now let's look at some of the laboratory tests used to evaluate the coagulation proteins. We'll first look at proteins in the "extrinsic" system. Current thinking considers the extrinsic system to be more physiologically important in clotting than its counterpart, the intrinsic system. The term extrinsic implies that the activation of Factor X to Factor Xa requires a substance not normally present in the circulation. That substance, shown here, is tissue factor or tissue thromboplastin. Tissue factor is a lipoprotein found on the vessel wall beneath a protective layer of endothelial cells. Injury to the vessel exposes tissue factor, releasing it into the circulation where it forms a complex with Factor VII. Tissue thromboplastin, Factor VII, and calcium ions quickly convert Factor X to Factor Xa.

Let's review the entire process. Upon injury to a vessel, the following things occur:

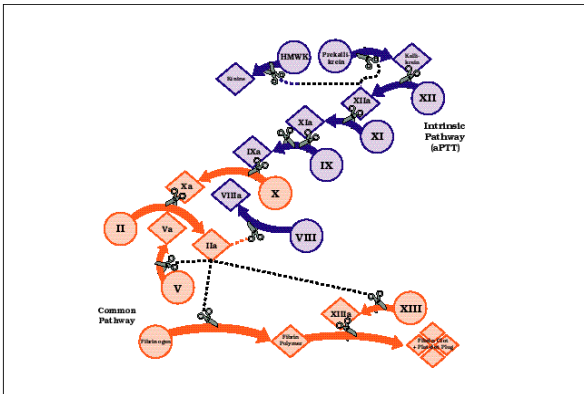
1. Tissue Factor is released.
2. Tissue Factor, along with Factor VII and calcium ions, forms a complex that activates Factor X to Factor Xa.
3. Factor Xa forms a complex with Factor V, calcium ions, and phospholipids to convert Prothrombin to Thrombin.
4. Thrombin converts Fibrinogen to Fibrin.

By using this information, we have the basis for an "in-vitro" screening test to assess the general activities of five circulating coagulation proteins (Factors I, II, V, VII, and X). This test is referred to as the Prothrombin Time or the Quick Time after Dr. Armand J. Quick who first described this assay.



Slide 32

The Prothrombin Time (PT) or Protime is performed by adding tissue thromboplastin to the patient's plasma. If a plasma sample exhibits a prolonged clotting time, we can say there is a deficiency or an inhibitor of one or more of the coagulation proteins, Factors I, II, V, VII, or X. Therefore, the Prothrombin Time is a widely used, simple screening test for deficiencies in the "extrinsic system". What cannot be said is which specific protein is at fault when a prolonged time is observed. By modifying this test system, we can determine the specific activity level of Factors II, V, VII, or X.

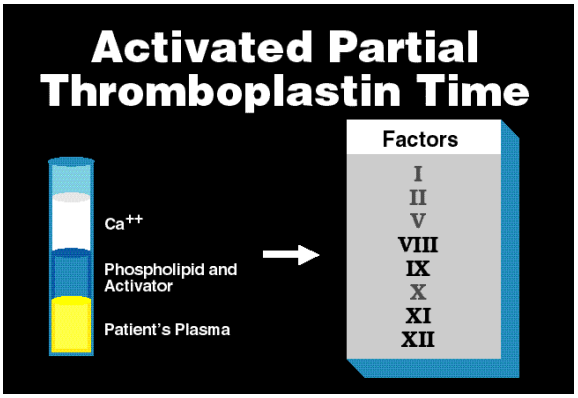


Slide 33

We've seen how Factor X is activated via the extrinsic pathway, but activation can also occur via the intrinsic pathway. The main difference between the two pathways is the rate of Factor Xa formation. The term "intrinsic" implies the involvement of substances normally present in the circulation. In the intrinsic pathway, Factor X is converted to Factor Xa by the enzyme Factor IXa, complexed with accessory factors, phospholipids, Factor VIII, and calcium ions. Factor IXa is formed from its naturally occurring plasma protein precursor, Factor IX, via the interaction of Factor XIa, calcium, and phospholipids.

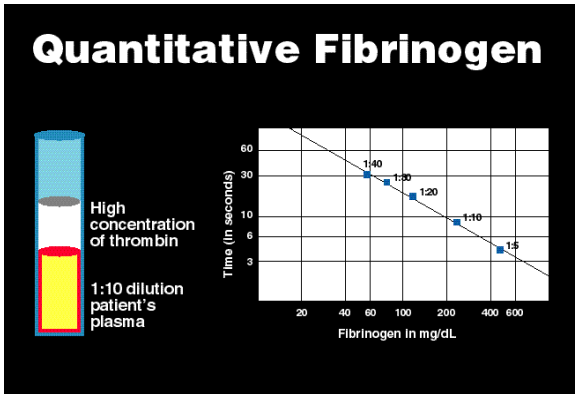
Factor XI is converted to Factor XIa in the presence of Factor XIIa. Likewise, Factor XIIa is derived from circulating Factor XII. In the initial phase of activation of the intrinsic system, Factor XII is converted to XIIa upon contact with a "foreign" surface, e.g. collagen or a vascular membrane exposed by injury.

Let's look at a summary of the intrinsic system. Beginning at the top, we see Factor XII going to XIIa in the presence of a foreign surface. Also note various endotoxins and some chemicals can activate Factor XII to Factor XIIa. Once this sequence begins, we can follow the cycle on down the slide. Factor XIIa activates Factor XI to Factor XIa; Factor XIa activates Factor IX to Factor IXa; Factor IXa along with Factor VIII, Platelet Factor 3 (phospholipids), and Ca^{++}, activates Factor X to Factor Xa and you know what happens when we have Factor Xa generated. It combines with Factor V, etc., prothrombin is converted to the active enzyme thrombin, and BOOM! fibrinogen is converted to fibrin – CLOT!

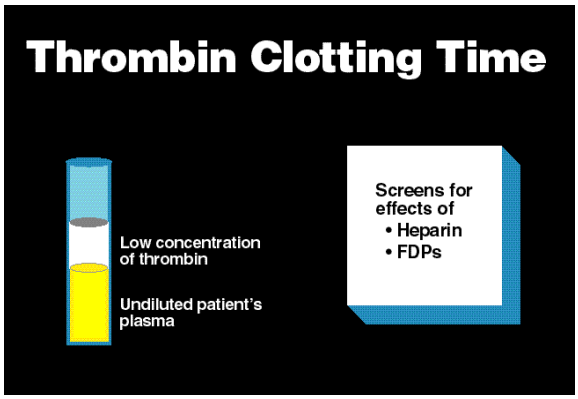


Slide 34

The intrinsic system can be evaluated *in vitro* using the Activated Partial Thromboplastin Time or APTT test. In this test, the intrinsic system is activated by adding phospholipid, activator and Ca^{++} to the patient's plasma. Materials such as ellagic acid, kaolin or micronized silica can be used to activate Factor XII, initiating the coagulation cascade and eventually converting fibrinogen to fibrin. The APTT system measures all the coagulation proteins with the exception of Factor VII and XIII. We'll later review how the APTT, like the PT, can be modified to determine activity levels of individual coagulation proteins. It is important to note that PT and APTT are global screening tests that provide a rough assessment of overall coagulation competence.

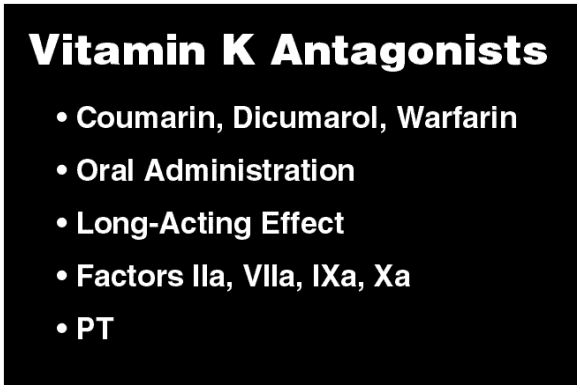


Slide 35
Neither PT nor APTT adequately screens for Factor XIII or fibrinogen deficiencies. PT and APTT detect conversion of fibrinogen to fibrin, therefore the cross-linking of fibrin by Factor XIII that occurs after this conversion does not impact the PT or APTT. As a rule of thumb, PT and APTT are unaffected by fibrinogen deficiencies until the level is below 50 mg/dL (normal range 200 to 400 mg/dL). The Clauss method for quantitating fibrinogen uses a high concentration of thrombin and a dilution of the patient's plasma. This bypasses the intrinsic and extrinsic pathways and dilutes any inhibitors or interfering substances, producing a quantitative result that reflects the patient's clottable fibrinogen. The clotting time is read against a standard curve.



Slide 36
The thrombin clotting time (TCT) procedure provides a simple screen for the presence of fibrin/fibrinogen degradation products (FDPs). Undiluted patient plasma and a low concentration of thrombin is used. We will discuss the effects of inhibitors and anticoagulants in more depth in later slides.

Slide 37
One of the primary reasons for routine utilization of PT and APTT assays is monitoring anticoagulant therapy. The two major classes of anticoagulants are Vitamin K antagonists and antithrombin-III (AT-III) accelerators. These anticoagulants are used to prevent thrombus formation and embolism. Heparin, an AT-III accelerator, is routinely used to prevent clot formation during cardiac surgical procedures.



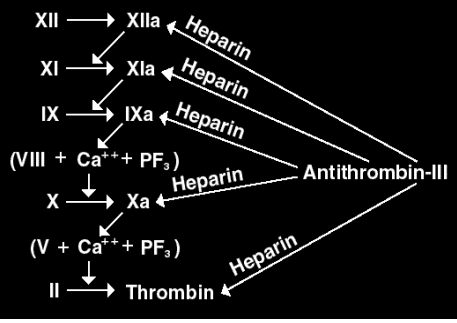
Slide 38
Coumarin derivatives are administered orally, usually following heparin therapy and for an extended period of time. These derivatives inhibit gamma carboxylation of the vitamin K-dependent coagulation factors (Factors IIa, VIIa, IXa and Xa). The inhibition prevents correct biosynthesis of active coagulation factors with subsequent production of inert molecules called PIVKAs (proteins induced by vitamin K antagonists). Coumarin therapy can be monitored by a number of techniques (prothrombin crossed immunoelectrophoresis, factor-specific assays), but the PT is most commonly used because of its simplicity, general availability and low cost.

AT-III Accelerators

- Heparin, Heparinoids, LMW Heparin
- Parenteral Administration
- Short-Acting Effect
- Factors IIa, IXa, Xa
- APTT

Slide 39
Heparin is the most commonly used AT-III accelerator. It is a highly acidic mucopolysaccharide. Commercial heparin may be of bovine or porcine origin with a molecular weight of about 25,000 to 35,000 daltons. Only about 1/3 of the heparin molecule is active, providing 85% of the anti-coagulant effect. Low Molecular Weight (LMW) heparins, about 5,000 to 10,0000 daltons, have recently been introduced. The type of heparin used and the method of administration impact the patient's response to therapy. Since heparin affects Factors IIa, IXa and Xa, the APTT can be used to monitor therapy. The most effective test for monitoring LMW heparins is the chromogenic substrate Xa inhibition assay, since LMW heparins primarily inhibit Factor Xa.

Heparin/AT-III Complex



Slide 40
Heparin acts at multiple sites in the coagulation pathway. Small amounts of heparin in combination with AT-III (heparin cofactor) inhibit thrombin formation by inactivating Factor Xa and inhibiting conversion of prothrombin to thrombin. Once active thrombosis develops, larger amounts of heparin inhibit further coagulation by inactivating thrombin, thus preventing conversion of fibrinogen to fibrin. Heparin also prevents stabilization of fibrin clots by inhibiting activation of fibrin stabilizing factor (Factor XIIIa).

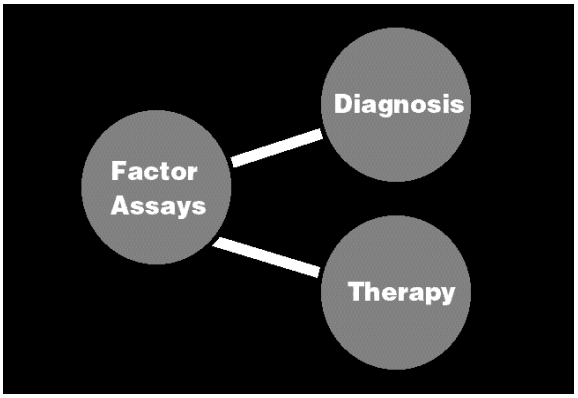
Variables in Monitoring Heparin

- Time of Specimen Collection
- Type of Anticoagulant
- Centrifugation of Specimen
- Instrumentation and Reagents

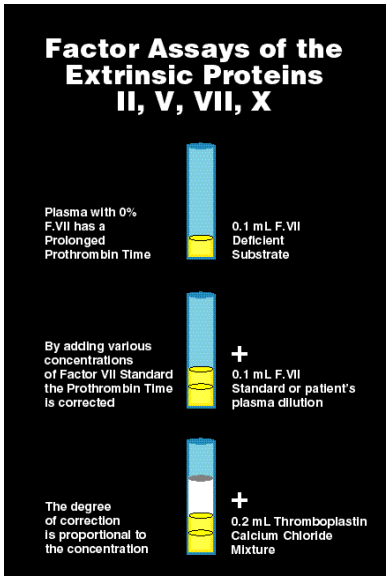
Slide 41
For testing to be meaningful, the variables in monitoring heparin therapy need to be considered. If possible, a baseline APTT value should be established prior to initiating therapy. The method of heparin administration determines timing of specimen collection – immediately prior to IV injection of the next dose, every 4 hours for IV infusion, and 4 to 6 hours after subcutaneous injection. Timing of specimen collection is particularly critical when the patient is being converted from heparin to coumarin therapy. A baseline PT value should be established by drawing a specimen when heparin activity is too low to affect PT (5 hours after last IV bolus or 24 hours after last subcutaneous dose).

Specimen handling is also important. Specimens should not be drawn with heparin syringes or into tubes containing heparin. Specimens must be centrifuged and the plasma separated immediately to prevent neutralization of the heparin by Platelet Factor 4 (heparin neutralizing factor) found in the buffy coat.

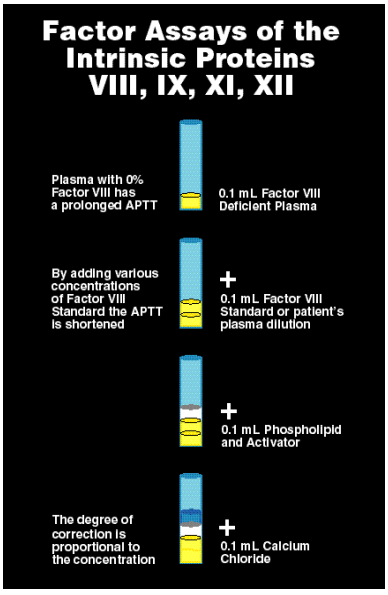
Consideration should also be given to the instruments and reagents used for the APTT assay.



Slide 42
While the PT and APTT are screening procedures, adaptations of these testing systems can be used to determine specific factor deficiencies. The coagulation proteins can also be measured directly using chromogenic substrate assays.

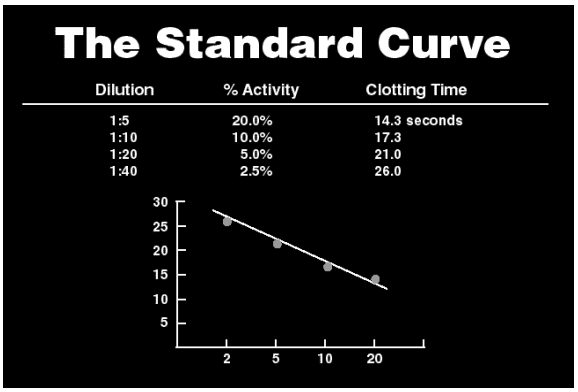


Slide 43
To determine activity levels of an individual protein, for example Factor VII, we must first have a plasma with no Factor VII present. This "deficient plasma" (<1% activity), will exhibit a prolonged clotting time. By adding various concentrations of Factor VII standard to the "deficient plasma" before adding the thromboplastin/calcium mixture, we will "correct" or shorten the clotting time. By plotting the clotting times versus the concentration of added Factor VII, we can construct a standard curve. Next, by comparing the unknown or patient plasma's ability to correct the clotting time of the deficient plasma to the degree of correction by the standard, we can determine the Factor VII activity of the unknown plasma.



Slide 44
Using a similar modification to the one employed in the Prothrombin Time procedure, we can assay the intrinsic coagulation proteins. Again, we start with a severely deficient plasma, for example, a hemophilic (Factor VIII deficient) plasma. This plasma alone will have a markedly prolonged APTT.

By adding dilutions of a known standard, we can correct the clotting time and prepare a standard curve. Next, we add dilutions of the unknown plasma and compare the clotting times to those of the standard. We read the % activity from the standard curve and multiply by the dilution factor, as shown in the next slide.



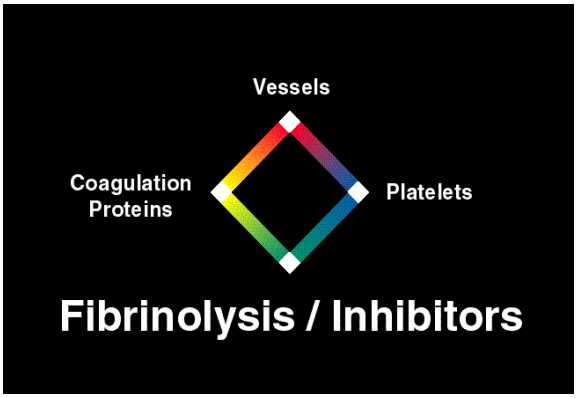
Slide 45

Factor assays are read against a standard. A Factor VII standard can be diluted as follows: 1:5, 1:10, 1:20, 1:40. We add 0.1 mL of the diluted standard to 0.1 mL of deficient plasma and perform a Prothrombin Time on the mixture. Here, using a semi-log graph, we plot the clotting time of the dilutions versus concentrations. The clotting time is on the Y axis and the % activity is on the X axis.

Using the standard curve, we can now calculate the unknown. Assume your patient's 1:10 dilution corrected the deficient plasma's clotting time to 19 seconds. Reading 19 seconds from the curve, we can see the % activity corresponding to 19 seconds reads about 8%. Multiplying 8% by 10 (the dilution factor), we arrive at a figure of 80%. If the Factor VII standard used to construct the curve was 100%, then our patient has a Factor VII activity of 80%. If the standard material was less than 100% activity (for example 90%), then we multiply 80% by .90 to arrive at the actual activity of the patient's plasma.

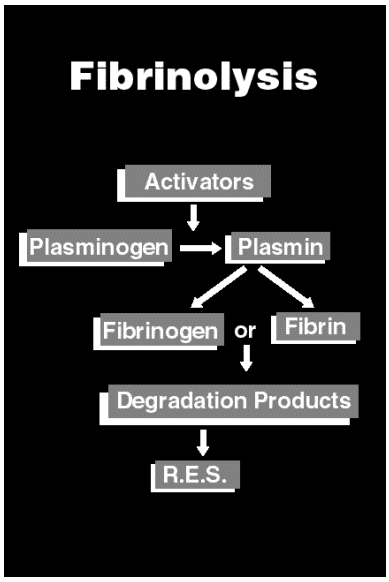
Slide 46

This list of effective hemostatic levels for single factor deficiencies has been extracted from the work of W. J. Williams (Hematology, 1972). The actual activity necessary for homeostasis is represented as a percentage of the normal level (except for fibrinogen which is expressed in mg/dL). As a general rule, spontaneous bleeding can be controlled if the patient's factor level is above the established minimum. For example, plasma levels of Factor VII above 5 to 10% of normal are sufficient to maintain homeostasis following minor trauma or during the healing period after surgery. On the other hand, Factor VII levels should be well above 10 to 20% before major surgery is contemplated, or if serious post-traumatic bleeding has occurred.



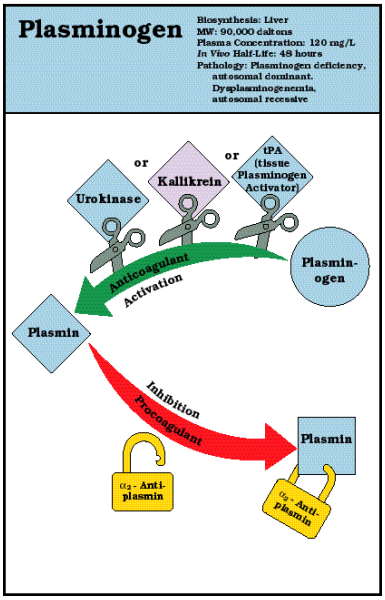
Slide 47

The fourth point of the diamond is the fibrinolytic/inhibitor system. The dynamics of hemostasis are such that thrombosis and thrombolysis are simultaneous events. The fibrinolytic/inhibitor system is critical to the maintenance of homeostasis. It is this system that prevents uncontrolled clot formation *in vivo*.



Slide 48

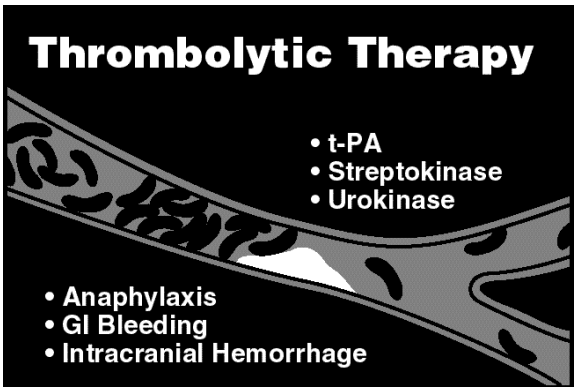
The key step in fibrinolysis is the conversion of plasminogen to the potent serine protease plasmin. Plasmin can digest either fibrinogen, fibrin monomers, or fibrin clots. The degradation products created in the process are removed from circulation by the reticuloendothelial system (RES). This is all a normal part of maintaining homeostasis. If, however, there is systemic activation of plasminogen and no fibrin to digest, plasmin will digest Factors I (fibrinogen), V and VIII. The fibrinogen degradation products (FDP) that result from fibrinogen digestion are antithrombotic in nature and inhibit polymerization of the clot. FDPs are easily detectable with laboratory tests and can be indicative of serious life-threatening conditions.



Slide 49

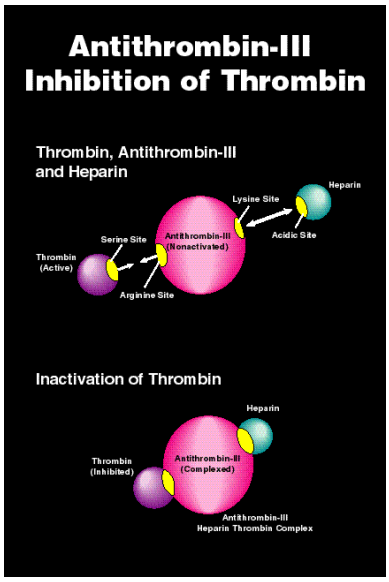
Plasminogen is structurally characterized by five molecular features called kringles, due to their resemblance to a Danish pretzel-like confection of the same name. One of these kringle structures (specifically K4) is repeated 37 times in apo(a), the apolipoprotein related to atherosclerosis.

Presumably, this structure confers the ability to bind fibrin, which is of course essential to direct the catabolic activity of plasmin against the fibrin clot. α_2-antiplasmin is yet another of the so called serpins (serine protease inhibitors) that are so important in regulating hemostasis.



Slide 50

Thrombolytic therapy is a pharmaceutical application of fibrinolysis which endeavors to open a vessel occluded by a thrombus. Urokinase (or the bacterial equivalent, streptokinase) and t-PA (tissue plasminogen activator) induce plasmin formation which dissolves the clot (as we saw on the previous slide). Thrombolytic therapy can cause serious complications: anaphylaxis, gastrointestinal bleeding and intracranial hemorrhage. Efforts are being made to implement tests that more clearly identify patients for whom thrombolytic therapy can be of benefit and to establish tests for monitoring thrombolytic therapy. Heparin and coumarin are useful therapeutic agents for preventing clot formation, however they have no lytic properties and cannot lyse a clot once it has been formed.



Slide 51
Antithrombin-III, also known as antifactor Xa and heparin cofactor, is the major inhibitor of thrombin and Factor Xa. Antithrombin-III by itself can bind and inactivate thrombin, but the presence of heparin increases the reaction rate multifold. Antithrombin-III, in complex with heparin, also inhibits Factors IXa, XIa, XIIa and prekallikrein, but at a slower rate.

Antithrombin-III has an *in vivo* half-life of about 2½ to 5 days and a normal plasma level of 13 to 20 mg/dL.

Antithrombin-III Decreased Levels

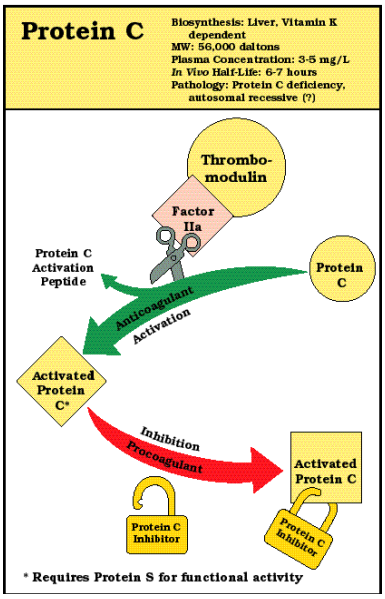
1. Congenital
2. Acquired – decreased synthesis
3. Acquired – increased utilization
4. Drug-induced

Slide 52
Antithrombin-III levels below 70% of normal can be clinically significant – responsiveness to heparin is impaired. We can divide decreased AT-III levels into several categories. Congenital deficiencies are autosomal dominant and predispose the patient to thrombotic episodes. The second category is decreased synthesis of antithrombin-III associated with disease conditions such as chronic hepatitis, adult onset diabetes, arteriosclerosis, cirrhosis and cardiovascular disease. In the third group of disorders – disseminated intravascular coagulation, myocardial infarction, post-operative states, nephrotic syndrome and pulmonary embolism – decreased antithrombin-III levels result from increased utilization (consumption). In the fourth category, drugs, such as oral contraceptives, heparin (constant infusion), fibrinolysin and L-asparaginase, can decrease antithrombin-III levels.

Protein C

- Vitamin K-dependent plasma protein
- Inactivates Factors V and VIII
- Stimulates fibrinolysis

Slide 53
Protein C is a Vitamin K-dependent protein with fibrinolytic and inhibitory properties. It is extensively involved in inhibition of Factor Va and Factor VIIIa, inactivating these factors by degradation. Protein C also functions as a stimulator of fibrinolysis.



Slide 54
One of the feedback control mechanisms that kicks in when thrombin is created, via the endothelial cell cofactor thrombomodulin, is the activation of Protein C. Activated Protein C destroys Factor Va and Factor VIIIa, but only in the presence of Protein S. The level of available ("free") protein S varies widely as the complement binding protein (C4b) concentration changes. C4b binding protein normally binds about 60% of total Protein S, preventing it from acting as a Protein C cofactor.

Deficiencies of Protein C

I. Congenital
Hereditary autosomal dominant

II. Acquired
A. DIC
B. Liver disease
C. During post-operative period
D. Anticoagulant therapy

Slide 55
Protein C deficiencies can be congenital or acquired. Congenital deficiency of Protein C is a rare autosomal dominant disorder. Protein C deficiency is more often acquired as a consequence of increased utilization in disseminated intravascular coagulation (DIC) and post-operative recovery. Deficiencies are also seen in liver disease and oral anticoagulant therapy, since Protein C is a vitamin K-dependent protein.

Clinical Manifestations

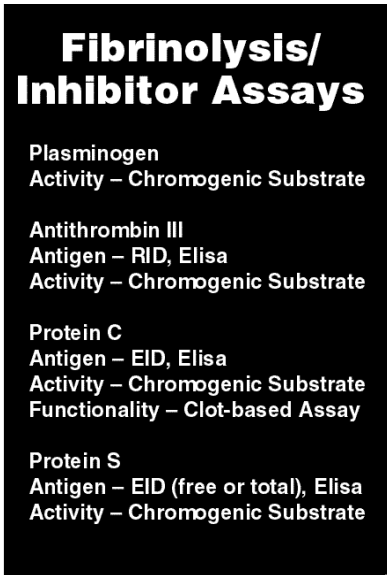
- Superficial thrombophlebitis
- Venous thromboses in adolescents or young adults
- Arterial thromboses rarely observed
- Skin necrosis during onset of oral anticoagulant therapy

Slide 56
Superficial thrombophlebitis and venous thrombosis in adolescents and young adults are the most common clinical manifestations of Protein C deficiency. Arterial thrombosis is not typically seen. Oral anticoagulant therapy can cause skin necrosis as a result of depressed levels of Protein C. This negative side effect is one reason some clinicians are turning from oral anticoagulants to alternate therapies such as aspirin.

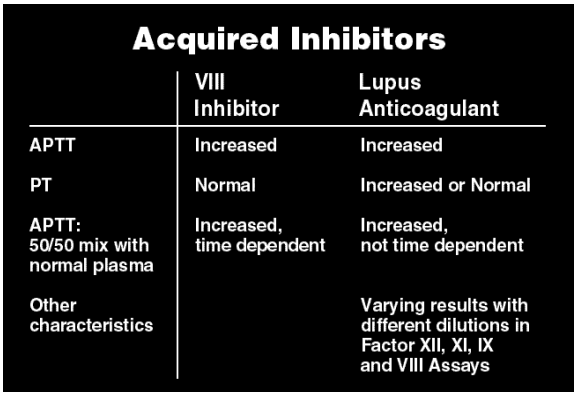
Protein S

- Cofactor for Protein C
- Vitamin K-dependent protein
- Enhances binding of Protein C to phospholipid surfaces

Slide 57
As previously mentioned, Protein S is a cofactor for Protein C and required for maximal expression of Protein C anticoagulant activity. Protein S can exist in two forms, free and bound. Anticoagulant inhibiting properties are only related to the free form. The free form complexes with Protein C, Ca^{++}, and phospholipids. It is this Protein C complex that acts to inhibit Factors Va and VIIIa by breaking these factors into inactive fragments.

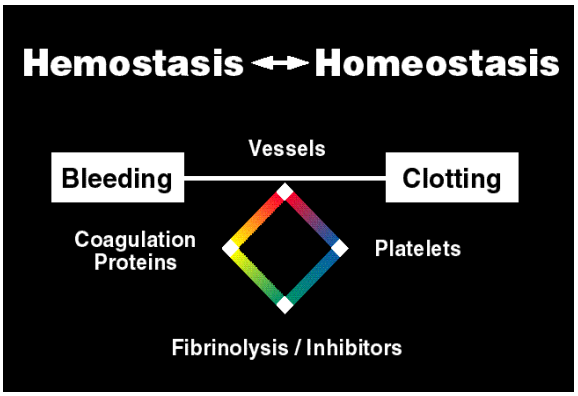


Slide 58
There are a number of assay procedures available for clinical evaluation of fibrinolytic and inhibitor proteins. The most commonly used methods rely on the antigenicity of the specific protein: radial immunodiffusion (RID), electroimmunodiffusion (EID), Elisa; or they measure activity (chromogenic substrate assays). Chromogenic substrate assays are available for plasminogen, AT-III, Protein C and Protein S. The Laurell rocket EID procedure for Protein S offers an advantage in that it can quantitate free Protein S.



Slide 59
Factor VIII inhibitor and Lupus-like anticoagulants are the two most common acquired inhibitors. Factor VIII inhibitor, as its name implies, specifically inhibits Factor VIII activity. The exact cause of this "acquired hemophilia" is not well understood, although it has been linked with transfusion, rheumatoid arthritis, pregnancy and certain drugs. The condition can be life threatening. The patient's APTT is prolonged and may partially correct when mixed with normal plasma. The most distinguishing characteristic is the marked increase in the APTT clotting time that occurs as mixed normal plasma incubates with the inhibitor-containing plasma.

Lupus anticoagulants are a class of phospholipid inhibitors associated with deep vein thrombosis and miscarriage. The patient's APTT may be prolonged, but does not markedly increase when the plasma is allowed to incubate. Its anticoagulant activity can produce erratic results with factor assays.



Slide 60
Seeing all the points of the homeostasis diamond gives us an appreciation for the complexities of balance and the consequences when there is an imbalance in any one part of the diamond. As our understanding of the vascular system, platelets, the coagulation proteins and the fibrinolytic/inhibitor system advances, we are increasingly aware of the importance of providing tests for the clinician that can give a complete picture of the patient's hemostatic status.

Routine Screening Tests

Coagulation System
PT – Extrinsic System
APTT – Intrinsic System
Fibrinogen

Platelet Studies
Count
Aggregation
Bleeding Time

Fibrinolysis / Inhibitors
Thrombin Clotting Time
FDP
Specific Assays –
AT-III, Protein C, Protein S

Slide 61
This combination of procedures, which most routine hemostasis laboratories can perform, enables the laboratory to provide clinicians with a comprehensive picture of the patient's hemostatic status. Testing includes procedures for coagulation, platelets and fibrinolysis. Undoubtedly, the future will bring changes to the profile of routine screening tests, including new tests for the vascular system.

Slide 62
The world was flat . . . until we ventured to the edge. The earth was our universe . . . until we left its pull to explore the heavens. Coagulation was a mystery . . . until we developed the tools to unravel its mystique. Our tools for understanding the complexities of hemostasis continue to evolve, and the things we are learning along the way are fascinating and potentially life-saving.

For more information on Helena's Hemostasis Systems, call toll free 800-231-5663.

Book W
2/93 (2)