Blood coagulation:

Blood is a necessary component of the human body, and the loss of this fluid may be life-threatening. *Hemostasis* (commonly known as blood coagulation; *heme* means *blood* and *stasis* means *to halt*) is the physiological process that stops bleeding at the site of an injury while maintaining normal blood flow elsewhere in the circulation. Blood loss is stopped by formation of a hemostatic plug. The endothelium in blood vessels maintains an anticoagulant surface that serves to maintain blood in its fluid state, but if the blood vessel is damaged, the components of the subendothelial matrix are exposed to the blood. Several of these components activate the two main processes of hemostasis to initiate the formation of a blood clot, composed primarily of platelets and fibrin. This process is tightly regulated so that it is activated within seconds of an injury but must remain localized to the site of injury. These processes have decidedly complicated mechanism and a detailed discussion is beyond the scope of this book. However, we will present a brief outline of these routes, particularly emphasizing on the chemistry behind them, whenever suitable for the audience of this text.

There are two main components of hemostasis. Primary hemostasis refers to platelet aggregation and platelet plug formation. Secondary hemostasis refers to the deposition of insoluble fibrin that forms a mesh which is incorporated into and around the platelet plug. This mesh serves to strengthen and stabilize the blood clot. These two processes happen simultaneously and are mechanistically intertwined. In essence, the clotting of blood is achieved by conversion of a soluble plasma protein fibrinogen to the insoluble, mesh-like cross-linked network of fibrins and this process is mediated by a *serine protease* called thrombin. Thrombin, however, is present in circulating blood as an inactive proenzyme prothrombin which needs to be activated by an *enzymatic cascade* involving a host of coagulation-promoting proteins called *clotting factors*.

Primary hemostasis involves activation of platelets in a multifaceted process, and as a result the activated platelets adhere to the site of injury and to each other, plugging the injury. The platelet plug is achieved in four phases: vasoconstriction, platelet adhesion, platelet activation, and platelet aggregation. In lieu of a detailed account of these processes which are reserved for specialized texts, we provide a gist of what happens here. Vascular injury results in the exposure of subendothelial collagen and von Willebrand factor (vWF). vWF is a large multimeric glycoprotein that serves as the initial stationary foundation on which a clot forms. It is secreted from endothelial cells and megakaryocytes that is always present in the soluble state in the plasma as well as in the immobilized state in subendothelial matrix. Subendothelial vWF binds to glycoprotein Ib (GpIb) on platelets. This causes a conformational change on the platelet surface that results in the exposure of glycoprotein IIb/IIIa (GpIIb/IIIa). Due to the conformational change, circulating fibrinogen attaches to GpIIb/IIIa. At this point in hemostasis, a soft platelet plug has formed, and the importance of biochemical interactions of clotting factors arises.

Secondary hemostasis refers to the deposition of insoluble fibrin, which, as mentioned earlier, is generated by the proteolytic coagulation cascade. Within secondary hemostasis, three coagulation pathways exist: intrinsic, extrinsic, and common. The intrinsic pathway responds to spontaneous, internal damage of the vascular endothelium whereas the extrinsic pathway becomes activated secondary to external trauma. Both intrinsic and extrinsic pathways meet at a shared point to continue coagulation, the common pathway. Clotting factors involved in the intrinsic pathway include factors XII, XI, IX, and VIII. Clotting factors involved in the extrinsic pathway include factors VII, and III. The common pathway includes clotting factors X, V, II, I, and XIII. Clotting factors can also be referred to outside of their Roman numeral designations. However, the initial naming of the clotting factors was arbitrary and sometimes confusing, as the same clotting factor were called by different names by different groups of workers. To bring

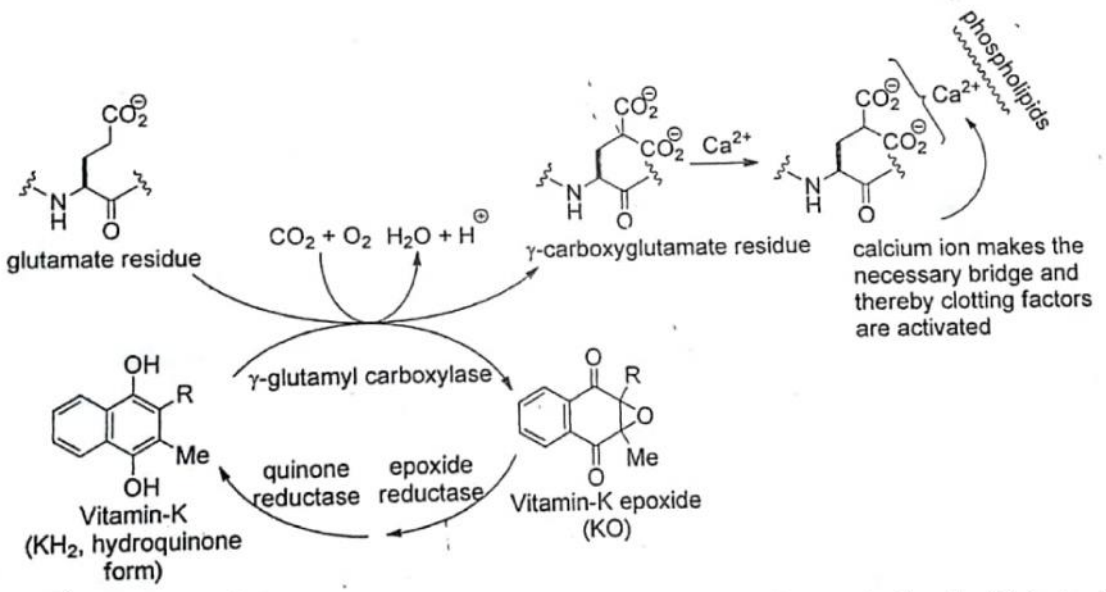
parity in this situation, these roman numerals were assigned to these factors in 1958. This nomenclature system has also evolved through the years and it is recommended now to refer to these molecules by the roman numerals. In some texts, still, some of the old names persist, so it is in order to familiarize oneself with at least the most popular synonym for each of these:

Secondary hemostasis Pathway	Clotting factor involved	Synonym
Intrinsic	XII	Hageman factor
	XI	plasma thromboplastin antecedent (PTA)
	IX	Christmas factor
	VIII	antihemophilic factor A
Extrinsic	VII	stabile factor / proconvertin
	III	tissue factor / thromboplastin
Common	X	Stuart-Prower factor
	V	proaccelerin
	II	prothrombin
	I	fibrinogen
	XIII	Fibrin-Stabilizing Factor (FSF)

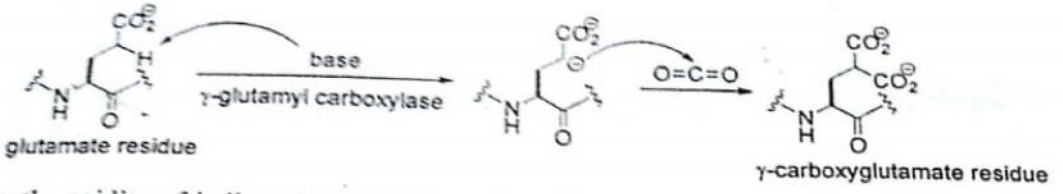
Note that there are no factor IV and factor VI. At one point of time Ca^{2+}, that plays an important role in all three pathways, was called the factor IV, but that use is archaic now. What was thought to be factor VI turned out to be the activated form of factor V and that is why it has been removed as well. Again some of the clotting factors are proenzymes (zymogens) to certain proteases, specifically factors II, VII, IX, and X, XI and XII. In addition to these, several other proteins and cofactors are associated with the coagulation pathway. We have listed in the above list only those that are necessary for the purpose of our discussion.

Before delving into the discussion of the secondary hemostasis pathways, let us address one important point relating to the binding of the clotting factors on the platelet surface at the site of the wound. In addition to the exposure of GpIIb/IIIa as a result of conformational change occurring on the platelet, the membrane phospholipid phosphatidylserine (PS) also emerges on the platelet surface. Activation of *scramblase* at the plasma membrane allows externalization of PS that is normally constrained within the cytosolic leaflet. Recall that the polar head group of

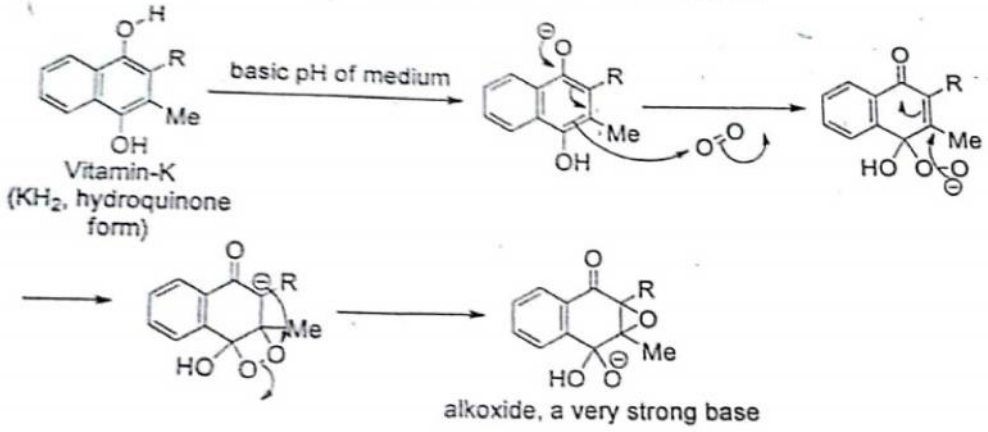
the PS has a negative charge. As a result, it provides an excellent surface for a Ca^{2+} ion to bind. The interaction between negatively charged PS and calcium does not completely negate calcium's positive charge. This allows for serine proteases to bind to the surface of the platelet membrane. This binding is possible due to carboxylation of clotting factors II, VII, IX, and X. These clotting factors contain regions where specific glutamic acid (glu) residues have been converted to γ-carboxyglutamic acid (gla) through a vitamin K dependent carboxylation, involving the enzyme γ-glutamyl carboxylase. The enzyme adds a negatively charged carboxyl group to glutamic acid residues (glu), which calcium easily binds to. As a result, the clotting factors can adhere to the platelet surface as *serine proteases*. This can be represented in the following way:



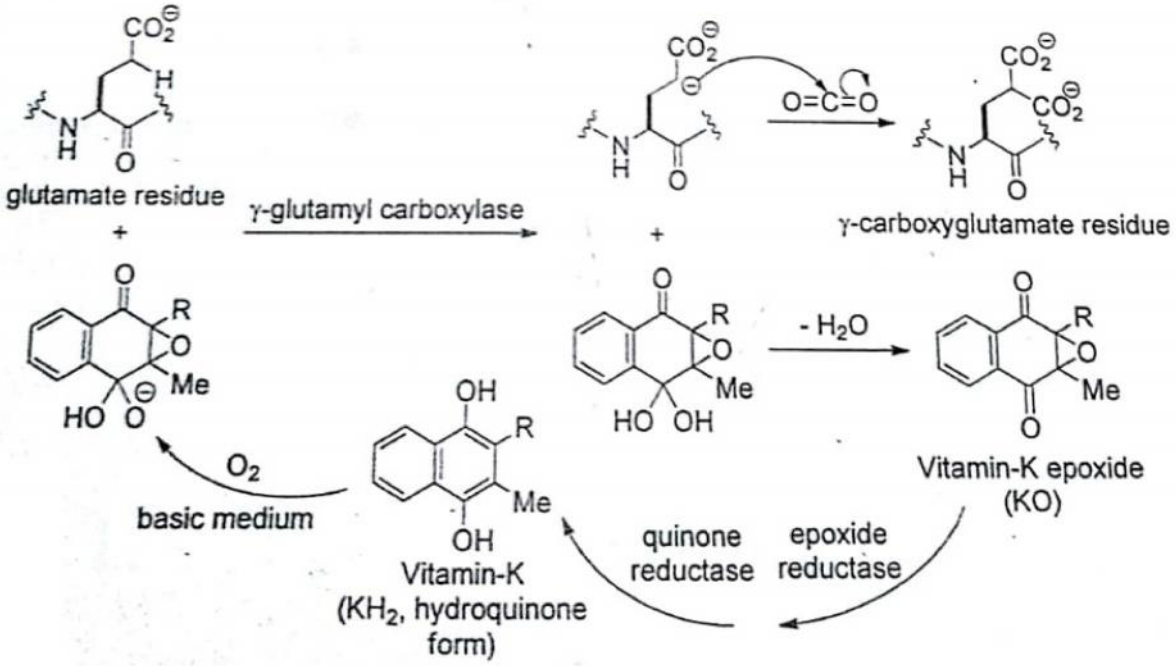
Before we part from this section an interesting chemistry of vitamin K is to be considered. Vitamin K was discovered by Henrik Dam in 1935.He was experimenting with cholesterol synthesis and observed that chicken fed with a cholesterol-deficient diet developed a coagulation disorder. The discovery of a vitamin that was obviously related to coagulation followed. The vitamin was called vitamin K since it has such a close relation to the process of *koagulation*, the Danish term for coagulation. However, we have pointed out that here that clotting factors contain regions where specific glutamic acid (glu) residues have been converted to γ-carboxyglutamic acid (gla) through a vitamin K dependent carboxylation, involving the enzyme γ-glutamyl carboxylase. Obviously for the carboxylation of glutamic acid to γ-carboxyglutamic acid (gla) a carbanion is to be generated from glutamic acid residue as indicated below:



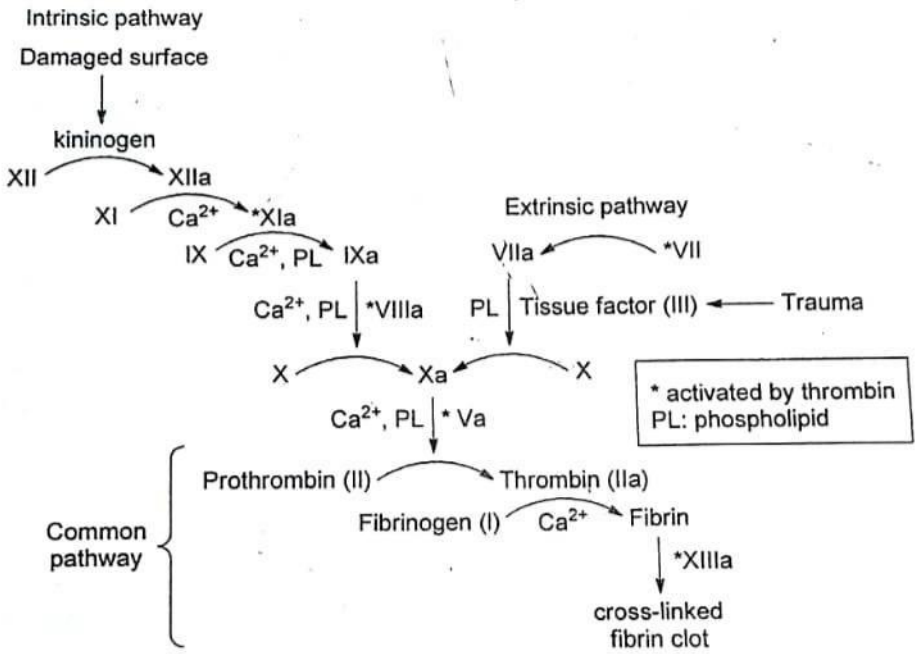
Now the acidity of indicated proton is very low (pk_a is around 27). Thus a very strong base is necessary to abstract that proton. Actually vitamin K is responsible to generate the necessary carbanion via the formation of a strong alkoxide base as shown below:



Thus the overall process for the formation of glutamic acid residue to γ-carboxyglutamic acid (gla) residue can be represented in the following way:



Let us now consider the pathways of secondary hemostasis. For convenience we present it in a simplified graphical format. In here the zymogen to enzyme activation is indicated with a letter "a". For example, factor XII activation is the first step of the intrinsic pathway. Its activation is induced via contact with subendothelial collagen in the presence of high molecular weight kininogen. In our scheme we represent this as conversion of XII to XIIa, which, in turn, activates XI into XIa, which leads to the activation of IX to IXa, and so on.



As can be seen from the above diagram, for the extrinsic pathway, coagulation is initiated when a membrane protein (tissue factor, clotting factor III) exposed to the bloodstream by tissue damage forms a complex with circulating factor VII or VIIa (factor VIIa is generated from factor VII by trace amounts of other coagulation proteases, including factor VIIa itself). The tissue factor–VIIa complex proteolytically converts the zymogen factor X to factor Xa. Factor Xa, with the aid of Va (a stimulatory protein) then converts prothrombin to thrombin, which, in turn, subsequently cleaves fibrinogen to form fibrin. The tissue factor-dependent steps of coagulation are called the extrinsic pathway because the source of tissue factor is extravascular. The extrinsic pathway is quickly damped through the action of a protein that inhibits factor VII once factor Xa has been generated.

The intrinsic pathway starts to operate with the activation of factor XII upon contact with subendothelial collagen in the presence of kininogen. This is why it is also sometimes called the contact pathway. It is called intrinsic because all its components are present in the

circulation. Enzymatic cascades eventually lead to Xa and then the common pathway is followed. The ensuing thrombin activates a number of components of the intrinsic pathway, including factor XI, a *protease* that activates factor IX, to maintain coagulation in the absence of tissue factor or factor VIIa. Thrombin also activates factors V and VIII, which are cofactors rather than proteases. Factor Va promotes prothrombin activation by factor Xa by as much as 20,000-fold, and factor VIIIa promotes factor X activation by factor IXa by a similar amount. Thus, thrombin promotes its own activation through a *feedback mechanism* that amplifies the preceding steps of the cascade. Factor XIII is also activated by thrombin. Factor XIIIa, which is *not* a serine protease, chemically cross-links fibrin molecules through formation of peptide bonds between glutamate and lysine side chains, which forms a strong fibrin network.

The sequential activation of zymogens in the coagulation cascade leads to a burst of thrombin activity, since trace amounts of factors VIIa, IXa, and Xa can activate much larger amounts of the irrespective substrates. Thrombin is also responsible for eventually triggering mechanisms that shut down clot formation, thereby limiting the duration of the clotting process and hence the extent of the clot. Such control of clotting is of vital physiological importance since the formation of even one inappropriate blood clot within an individual's lifetime may have disastrous consequences.

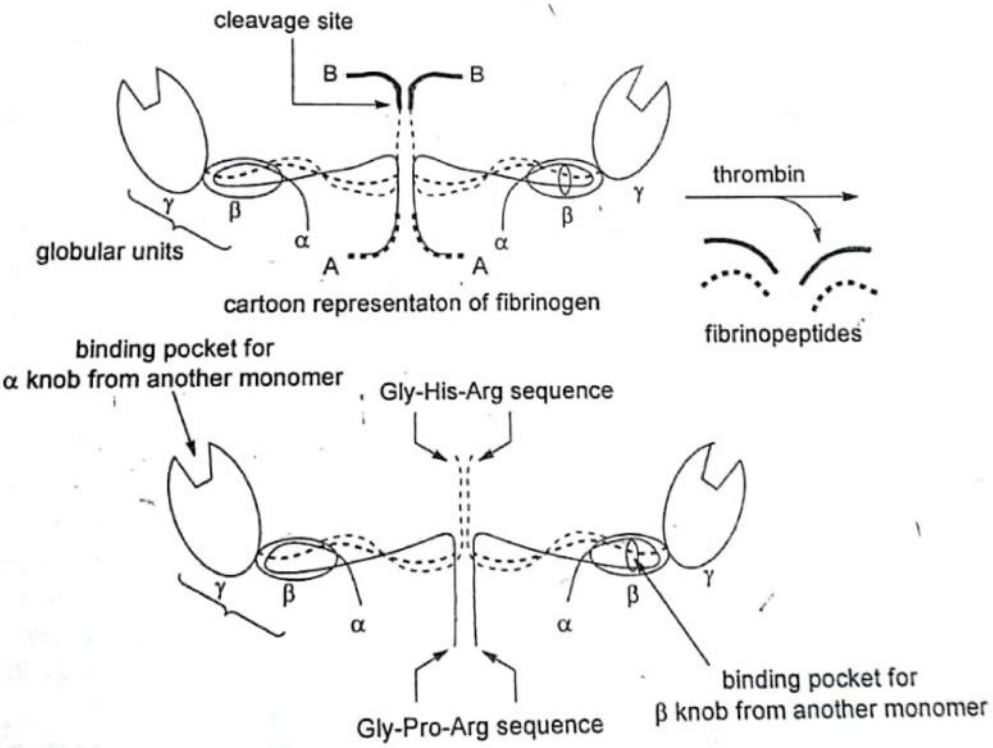
Extrinsic pathway happens much more quickly than intrinsic pathway. The intrinsic pathway of coagulation can be triggered by exposure to negatively charged surfaces such as glass. Consequently, blood clots when it is collected in a clean glass test tube. In the absence of tissue factor, a fibrin clot may not appear for several minutes, but when tissue factor is present, a clot forms within a few seconds. This suggests that rapid blood clotting *in vivo* requires tissue factor as well as the proteins of the intrinsic pathway.

As chemists, we are always interested in what happens at the molecular level and in this regard, the blood-clotting pathway has been a hotbed of research for many years. We will provide brief descriptions of the molecular events associated with two of the key steps of coagulation here.

1. Conversion of prothrombin to thrombin: This process involves binding of the gla domain, enriched in γ-carboxy glutamate residues, of prothrombin with Ca^{2+}, leading to anchoring of the protein onto the phospholipid (PL) membranes derived from blood platelets after injury. This anchorage of the zymogen brings it in close proximity of Xa and Va which proteolytically cleave it to thrombin. Activation is begun by proteolytic cleavage of the bond between arginine 274 and threonine 275 to release a fragment containing the first three domains. Cleavage of the bond between arginine 323 and isoleucine 324 yields active thrombin.

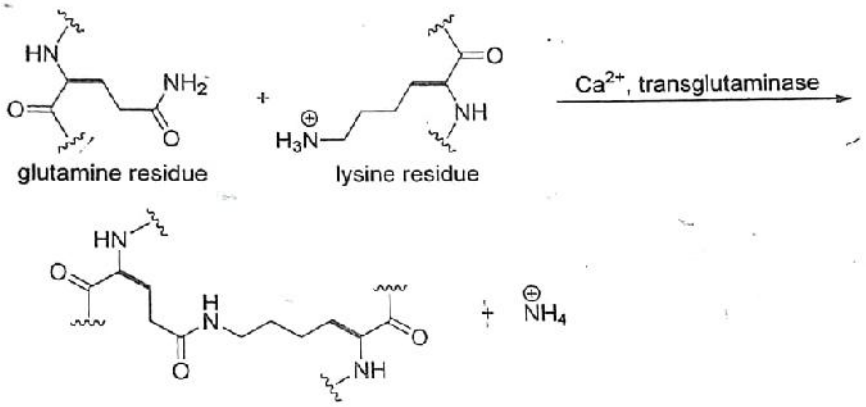
2. Conversion of fibrinogen into the fibrin clot by thrombin: This final step of the coagulation cascade is perhaps the best understood pathway of the entire clotting mechanism. Fibrinogen

is made up of three globular units connected by two rods. This 340-kDa protein consists of six chains: two each of Aα, Bβ, and γ. The rod regions are triple-stranded α-helical coiled coils. Thrombin cleaves four arginine–glycine peptide bonds in the central globular region of fibrinogen. On cleavage, an A peptide of 18 residues is released from each of the two Aα chains, as is a B peptide of 20 residues from each of the two Bβ chains. The peptides released A and B peptides are called fibrinopeptides. A fibrinogen molecule devoid of these fibrinopeptides is called a fibrin monomer. Fibrin monomers spontaneously assemble into ordered fibrous arrays called fibrin. Higher resolution images reveal how the removal of the fibrinopeptides permits the fibrin monomers to come together to form fibrin. The homologous β and γ chains have globular domains at the carboxyl-terminal ends. These domains have binding "holes" that interact with peptides. The β domain is specific for sequences of the form H_3N^+-Gly-His-Arg-, whereas the γ domain binds H_3N^+-Gly-Pro-Arg-. Exactly these sequences (sometimes called "knobs") are exposed at the amino-terminal ends of the β and α chains, respectively, on thrombin cleavage. This can be roughly represented as:



The knobs of the α subunits fit into the holes on the γ subunits of another monomer to form a protofibril. This protofibril is extended when the knobs of the β subunits fit into the holes of β subunits of other protofibrils. Thus, analogous to the activation of chymotrypsinogen, peptide-bond cleavage exposes new amino termini that can participate in specific interactions. The

newly formed "soft clot" is stabilized by the formation of amide bonds (isopeptide bonds) between the side chains of lysine and glutamine residues in different monomers. This cross-linking reaction is catalysed by *transglutaminase* (factor XIIIa). Ca^{2+} is a cofactor for this *fibrinoligase* action.



Contrary to the simplified secondary hemostasis pathways that we have presented above, it is now believed that intrinsic pathway does not simply run parallel to the extrinsic pathway, but indeed augments thrombin generation primarily initiated by extrinsic pathway. But discussion of these aspects are reserved for specialized texts.

Collection of Blood and Preservation:

In preparing a patient for phlebotomy, care should be taken to minimise factors related to activities that might influence laboratory determination. These factors include diurnal variation, exercise, fasting, diet, ethanol consumption, tobacco smoking, drug ingestion and posture. After 48 hours of fasting, serum bilirubin may increase. Fasting 72 hours decreases plasma glucose to 45 mg/dl, while a study in men showed an increase in plasma triglyceride, glycerol, and free fatty acids, with no significant change in cholesterol. When determine blood constituents e.g. glucose, triglyceride, cholesterol and electrolytes, collection should be in the basal state. Eating a meal depending on fat content, may elevate some constituents. In addition, physiological changes may include hyperchylomicronemia, thus increasing turbidity of the serum and plasma and potentially interfering with instrument readings. A high protein diet always increases serum urea, ammonia levels.

Again, there may be drug interference. Drug interferences are of two types: (a) physiologic *in vivo* effects of the drugs and its metabolites and (b) *in vitro* effects that result from some physical and/or chemical property that interfere with the assay.

Some physical attributes to consider when obtaining blood specimen include posture and incorrect tourniquet application. As a patient moves from supine to a standing position, there is a hydrostatic efflux of water and filterable substances from the intravascular space to the dependent interstitial fluid of the extracellular space. Non-filterable substances such as, protein, cellular elements and compounds associated with either, will increase in concentration in the intravascular space. Albumin and Ca^{2+} levels may become elevated as one changes position from supine to upright. Elements that are affected by postural changes are albumin, total protein, enzymes, calcium, bilirubin, cholesterol etc.

band fixed in upper limb before blood sample collection

Incorrect application of tourniquet and fist exercise can result in erroneous test results. Using a tourniquet to collect blood to determine lactate concentration may increase the lactate to falsely high levels. Prolonged tourniquet application may also increase serum enzymes, protein and protein-bound substances, including cholesterol, Ca^{2+}, and triglyceride. Stress, anxiety and hyperventilation may affect hormone secretions and acid-base balance. In general, patient scheduled for phlebotomy should refrain from strenuous physical activity, alcohol, drug or changes in diet for 24 hours prior to procedure.

Blood is the most frequent body fluid used for analytical purposes. Three general procedures for obtaining blood are: (a) Venipuncture, (b) arterial puncture and (c) skin puncture. The technique used to obtain the blood specimen is critical in order to maintain its integrity. Even so, arterial and venous blood differs in important respects. Arterial blood is essentially uniform in composition throughout the body, whereas the composition of venous blood varies and is dependent on metabolic activity of the perfused organ or tissue. Site of collection can affect the venous composition. Blood obtained by skin puncture (incorrectly called capillary blood) is an admix of blood from arterioles, venous and capillaries. Skin puncture blood also contains interstitial and intracellular fluids. However usually for common biochemical tests blood is collected through venipuncture.

Technique for Venous puncture: Usually a suitable vein of left arm is selected for puncture. Veins of the antecubital fossa, in particular, the median cubital and cephalic veins are preferred. Veins of wrist, ankle and hand may also be used. If one arm has an intravenous line, use the other arm to draw a blood specimen. At first the venepuncture site is cleaned with 70% isopropanol alcohol solution or 1% iodine-saturated swab stick. Then a tourniquet is applied at several inches above the puncture site only for 1 minute. Then the needle of syringe at approximately 15° angle to the arm is inserted into the vein. The geography of the vein is followed with the needle. The tourniquet is released when blood begins to flow. Capillary blood

may be collected from the tip of the thumb or finger or from the ear lobe by using a sterile needle.

Preservation of samples: Alteration in the concentration of a constituent in a stored specimen can result from various processes such as:

- Adsorption on to the glass container.
- Evaporation if the constituent is volatile.
- Water shift due to the addition of anticoagulants.
- Metabolic activities of the erythrocytes and leucocytes (accelerated by haemolysis) inducing O_2 consumption and CO_2 production, hydrolysis, glycolysis and finally degradation.
- Microbial (fungal/bacterial) growth.

Changes in concentration of volatile substances such as O_2 and CO_2 are prevented or at least hindered by collection and storage of samples under anaerobic conditions. The problem of microbial growth appears when the sample is to be stored for longer than one day either at room or refrigerator temperature. This can be solved by four alternative courses of action:

- Collection and storage under sterile conditions.
- Freezing of the sample.
- Extreme alteration of pH.
- Addition of an antibacterial agent.

Lyophilized samples are stable with respect to many constituents for periods of at least as long as ten years. Samples can be stored at room temperature (18-37°C), refrigerator temperature (4°C) and frozen state (-10°C or lower). With few exceptions, lower the temperature increases the stability. Further, microbial growth is considerably less at refrigerator temperature than at room temperature and is completely inhibited in the frozen state. Even in the frozen state, however, some components of plasma deteriorate.

Often after collection of blood it is preserved by using chemical preservatives. These can be classified into two groups:

- For prevention of chemical changes such as glycolysis
- For prevention of microbial growth.

Sander in 1923 introduced the combination of 10 mg Sodium fluoride + 1 mg thymol / mL of blood for preservation. The presence of thymol effectively controlled microbial growth so that non-sterile specimens are stable for all determinations (except non-protein nitrogen) for at least

two weeks. Chlorobenzene and bromobenzene have also been coupled with fluoride and have been claimed to be superior to thymol. Antibiotics can be used to prevent bacterial growth. We have already pointed out that potassium oxalate is often used as anticoagulant. Here oxalate ion forms the insoluble calcium oxalate which precipitates from the medium, thereby stopping the coagulation cascade. It is used together with sodium fluoride which inhibits glycolysis in collected blood. Among others, ethylenediamine (EDTA) and citrate also chelate calcium and prevent clotting. In this context it may be informed that the common preservatives for urine specimen are formaldehyde, thymol, toluene and chloroform. All these act primarily as antimicrobial agents.

To carry out precise estimation, dilution of blood sample is often necessary. For this purpose, normal saline is used but not the distilled water. If we use distilled water to dilute blood, then all the red blood cells explode. The blood becomes hemolysed. This is because of a process called 'osmosis'. When we separate two solutions, with different amounts of solutes dissolved in them, by a semi-permeable membrane, then water will pass across the membrane from the more dilute side to the more concentrated side. The red cell's membrane is semi-permeable; and its inside has a lot of substances dissolved in it. So if we put a cell into distilled water then the water will pass, by osmosis, from the water into the cell, increasing its volume until it bursts. Normal saline is made to be 'isotonic' (same osmotic potential) as blood plasma to avoid this happening.

Anemia:

Anemia (also spelled anaemia) is a blood disorder in which there is a deficiency of actual or available haemoglobin in the blood. Oxygen saturation is often normal but the reduced oxygen capacity leads to oxygen deficiency. Red blood cells has great role in anemia. Actually red blood cells carry haemoglobin, an iron-rich protein that attaches to oxygen in the lungs and carries it to tissues throughout the body. Anemia occurs when there is decreased number of RBC in the blood or when red blood cells do not function properly. It is diagnosed when a blood test shows a haemoglobin value of less than 13.5 gm/dl in a man or less than 12.0 gm/dl in a woman. Anemia can be temporary or long term, and it can range from mild to severe. Anemia is associated with the symptoms of weakness, shortness of breath, dizziness, fast or irregular heartbeat, pounding or "whooshing" in ears, headache, cold hands or feet, pale or yellow skin, chest pain.

The causes of anemia may be classified as impaired red blood cell (RBC) production, increased RBC destruction (hemolytic anemia), blood loss and fluid overload (hypervolemia). Several of these may interplay to cause anemia. The most common cause of anemia is blood loss, but this usually does not cause any lasting symptoms unless a relatively impaired RBC production develops, in turn most commonly by iron deficiency. Anemia can be classified into the following types:

- *Iron-deficiency anemia* is the most common type of anemia caused by the inadequate amount of iron in the body.

- *Vitamin-deficiency anemia* may result from low levels of vitamin B_{12} or folate (folic acid), usually due to poor dietary intake. Pernicious anemia is a form of megaloblastic anemia due to vitamin B_{12} deficiency dependent on impaired absorption of vitamin B_{12}.

- *Aplastic anemia* is a rare, life-threatening, bone marrow failure disorder in which the bone marrow stops making enough blood cells (red blood cells, white blood cells, and platelets). This type of anemia occurs as a result of destruction or deficiency of blood-forming stem cells in bone marrow, in particular, when the body's own immune system attacks the stem cells. Causes of aplastic anemia include infections, certain medicines, autoimmune diseases and exposure to toxic chemicals.

- *Hemolytic anemia* occurs when red blood cells are destroyed faster than bone marrow can replace them. Hemolytic anemia may be due to mechanical causes such as leaky heart valves or aneurysms, infections, autoimmune disorders, or congenital abnormalities in the red blood cell.

- *Sickle cell anemia* is an inherited hemolytic anemia. It is caused by a defective form of haemoglobin that forces red blood cells to assume an abnormal crescent (sickle) shape. These irregular blood cells die prematurely, resulting in a chronic shortage of red blood cells.

- *Anemia can also be caused by other diseases.* Some diseases can affect the body's ability to make red blood cells. For example, some patients with kidney disease develop anemia because the kidneys are not making enough of the hormone erythropoietin to signal the bone marrow to make new or more red blood cells. Chemotherapy used to treat various cancers often impairs the body's ability to make new red blood cells, and anemia often results from this treatment.