Proteolytic Activation of Zymogens in Blood Clotting

(A Further Look at Activation by Cleavage: Blood Clotting

The activation of zymogens is a fundamental step in vertebrate blood clotting, in which soluble plasma proteins are converted into an insoluble fibrin network that stops bleeding.

When a blood clot is examined under the electron microscope, as shown in Figure 11.55, it appears as striated fibrin fibers. Each fibrin monomer is an elongated molecule approximately 46 nm in length, and these monomers associate with neighboring molecules in a staggered arrangement.

Fibrin is formed from its precursor fibrinogen through proteolytic cleavage that releases fibrinopeptides A and B, exposing binding sites that allow fibrin molecules to aggregate.

After the fibrin network is established, it is further stabilized by covalent cross-links between glutamine and lysine residues catalyzed by Factor XIII (13), which strengthens the clot.

Thrombin, a highly specific serine protease structurally related to trypsin, catalyzes the conversion of fibrinogen to fibrin by cleaving specific Arg–Gly bonds. Thrombin itself is produced from prothrombin (Factor II) through a series of proteolytic activation reactions known as the coagulation cascade.

Most clotting factors, once activated, belong to the family of serine proteases classified under EC 3.4.21.x, including Factors XIIa (12), XIa (11), IXa (9), Xa (10), and VIIa (7), along with thrombin (Factor IIa), plasmin, and t-PA.

The major exception is Factor XIII (13), which is a transglutaminase rather than a serine protease.

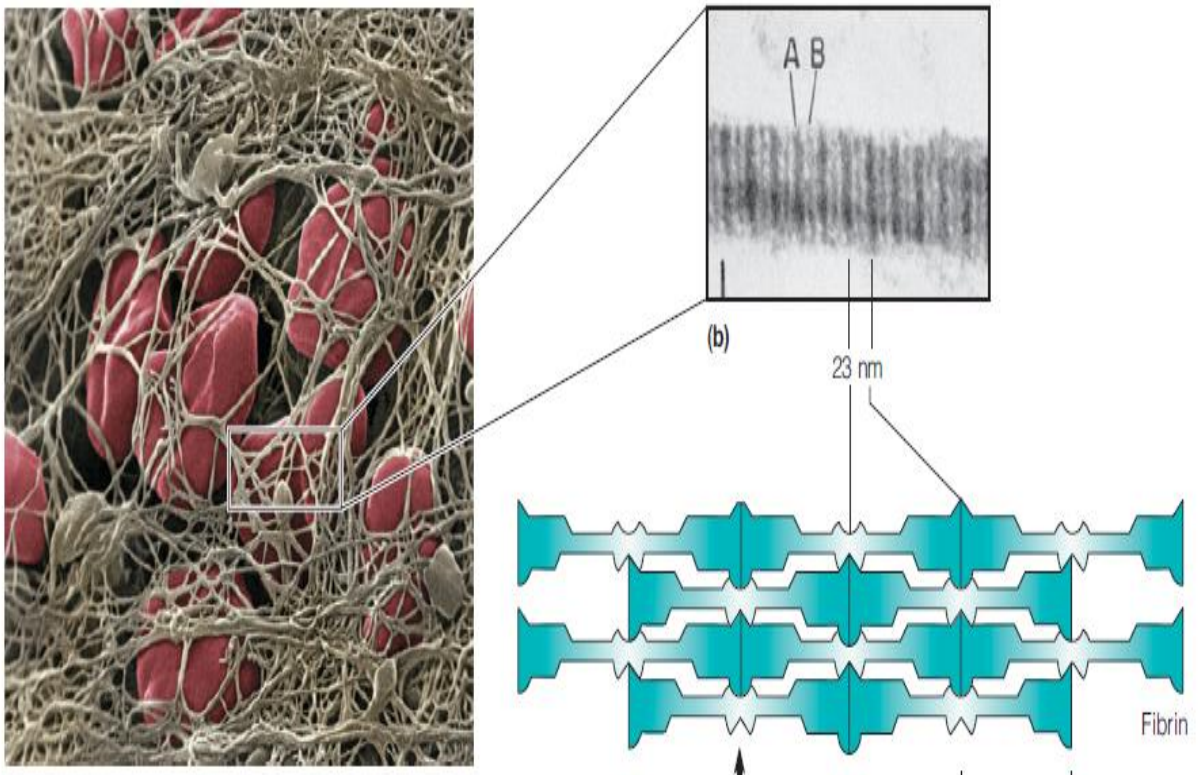


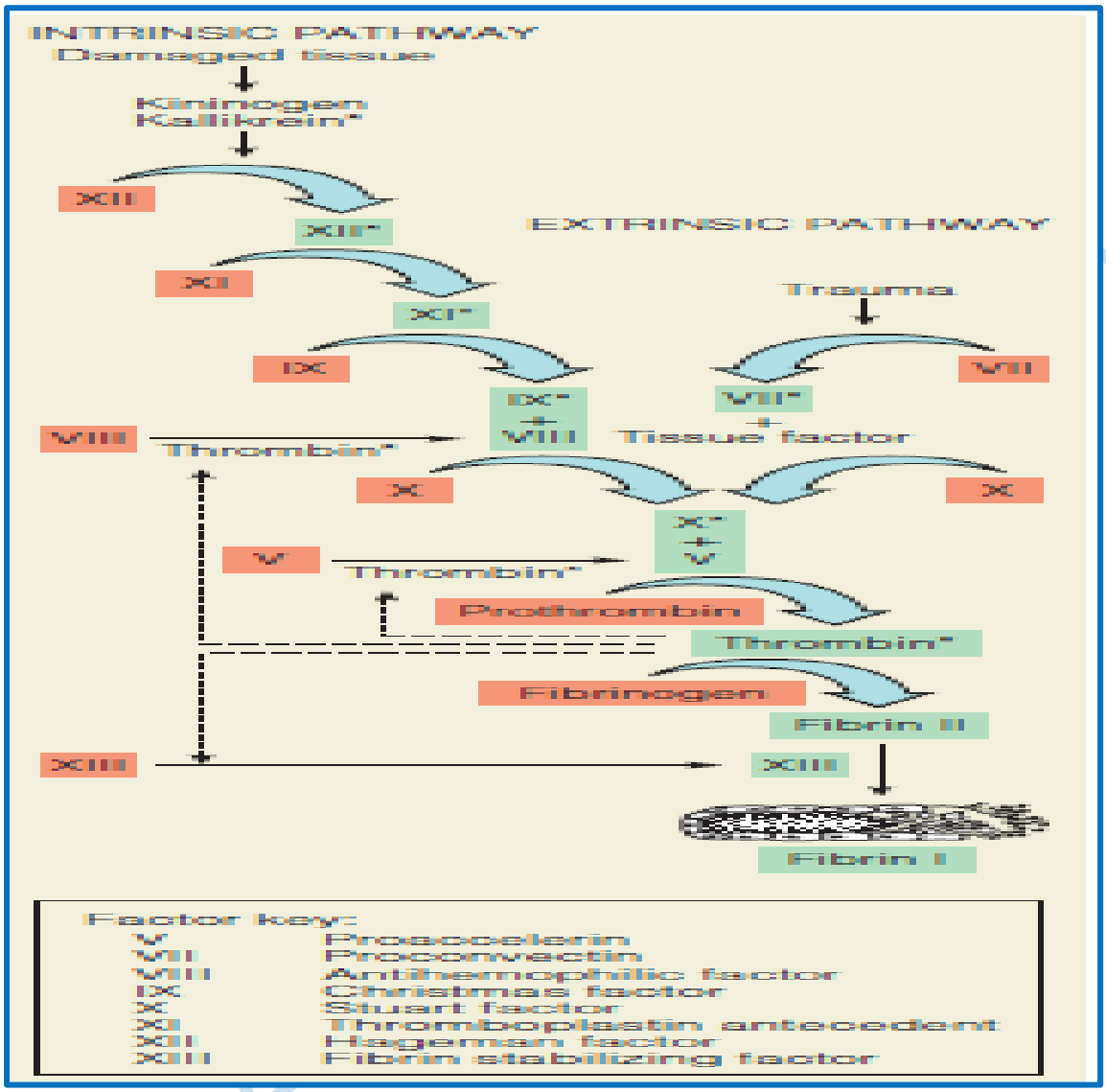
FIGURE 11.55

Formation of a blood clot. (a) Red blood cells enmeshed in the insoluble strands of a fibrin clot. **(b)** Electron micrograph of part of a fibrin fiber. **(c)** Schematic view of how fibrin monomers are thought to associate to form a fiber. Removal of fibrinopeptides A and B from fibrinogen by thrombin makes sites accessible for association with complementary sites a and b on adjacent monomers. The molecules are believed to overlap as shown because the striations seen in the

Figure 11.56 illustrates the sequence of reactions in the clotting cascade. In the intrinsic pathway, activation begins with Factor XII (12), followed by Factor XI (11) and Factor IX (9), with Factor VIII (8) serving as an essential cofactor to activate Factor X (10).

In the extrinsic pathway, damage to blood vessels leads to the release of Tissue Factor, which activates Factor VII (7). Both pathways converge at the activation of

Factor X (10), which converts prothrombin (II) into thrombin, initiating fibrin formation, which is later stabilized by Factor XIII (13).



After the clot has performed its essential role in preventing blood loss, it must be removed to restore normal blood flow. This process, known as fibrinolysis, depends on plasmin, an enzyme that digests fibrin. Plasmin is generated from its inactive precursor plasminogen through proteolytic activation by tissue-type plasminogen activator (t-PA). Clinically, t-PA is highly effective in dissolving unwanted blood clots, such as those responsible for stroke or myocardial infarction.

Note: Hemophilia is classified based on the deficient clotting factor. Hemophilia A results from a deficiency of Factor VIII (8), Hemophilia B from a deficiency of Factor IX (9), and Hemophilia C from a deficiency of Factor XI (11).

Molecular Engineering of New and Modified Enzymes

New or radically modified enzymes can be created by "protein engineering," which includes a number of techniques such as site-directed mutagenesis, protein domain fusion, selection from randomly generated libraries, and computational design.

Several approaches are being taken to achieve the goal of generating such as:

1- Tailor-made catalysts. These include site-directed mutagenesis,
2- Fusion of one or more functional domains,
3- Selection of a sequence with the desired activity from a large pool of randomly generated protein sequences,
4- Generation of "catalytic antibodies," and
5- Computational design.

NOTE: Tools of Biochemistry 11B provides a brief introduction to these techniques.

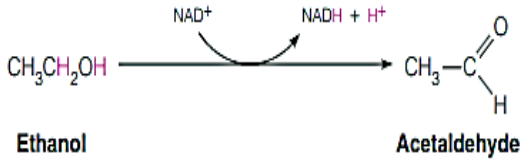
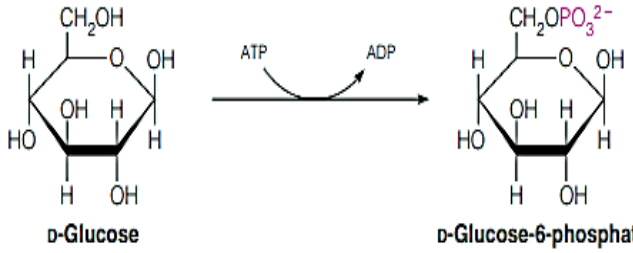
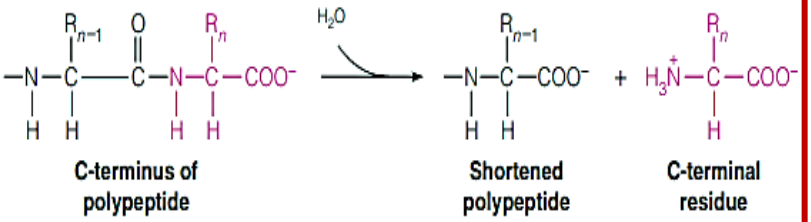
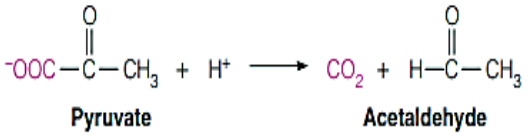
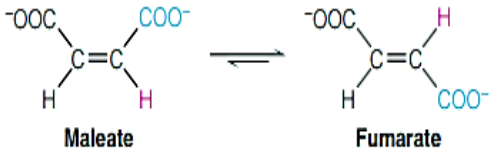
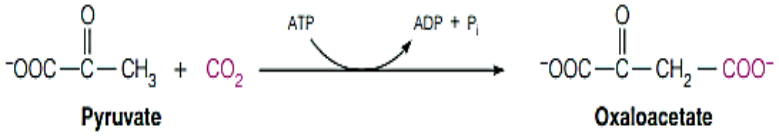
International Union of Biochemistry and Molecular Biology (IUBMB) Enzyme Commission (EC)

The IUBMB Enzyme Commission (EC) has given each enzyme a number with four parts, such as:

EC 3.4.21.5. The first three numbers define major class, subclass, and sub-subclass, respectively. The last is a serial number in the sub-subclass, indicating the order in which each enzyme is added to the list, which is continually growing.

Table 11.10 lists one example enzyme and reaction from each of the major classes.

TABLE 11.10 Examples of each of the major classes of enzymes

Class	Example (reaction type)	Reaction Catalyzed
1. Oxidoreductases	Alcohol dehydrogenase (EC 1.1.1.1) (oxidation with NAD^+)	
2. Transferases	Hexokinase (EC 2.7.1.2) (phosphorylation)	
3. Hydrolases	Carboxypeptidase A (EC 3.4.17.1) (peptide bond cleavage)	
4. Lyases	Pyruvate decarboxylase (EC 4.1.1.1) (decarboxylation)	
5. Isomerases	Maleate isomerase (EC 5.2.1.1) (*cis–trans* isomerization)	
6. Ligases	Pyruvate carboxylase (EC 6.4.1.1) (carboxylation)	

TOOLS OF BIOCHEMISTRY

How to measure the rates of enzyme-catalyzed reactions?

There are essentially two approaches to enzyme kinetic analysis.

1. **Steady-state analysis**, where the Michaelis–Menten equation applies, and reaction velocity is measured as a function of substrate and enzyme concentrations.
2. **Pre–steady–state analysis**, used to study very fast, early steps of the reaction mechanism and requiring specialized rapid techniques.

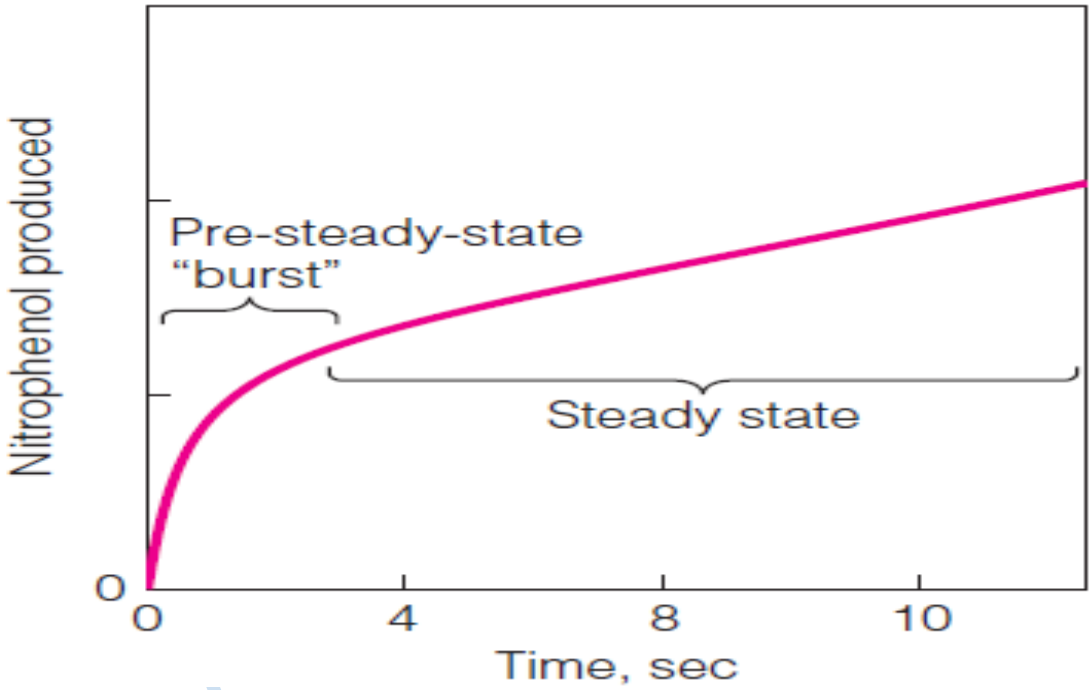


FIGURE 11.27
The pre-steady-state. This plot shows the kinetics of chymotrypsin-catalyzed hydrolysis of *p*-nitrophenyl acetate. The production of the first product (p-nitrophenol) is monitored spectrophotometrically after the enzyme and substrate are mixed. The initial burst of product formation ceases when the enzyme is almost all bound in the acyl–enzyme intermediate.

Analysis of the Steady State

The steady state is generally reached within seconds or minutes and persists long enough to be measured easily. Several experimental techniques are commonly used:

1. Spectrophotometry

A simple and accurate method that requires a substrate or product with a unique absorbance spectrum.

Example: Following NADH formation or consumption at 340 nm.

2. Fluorescence

Like spectrophotometry, but often more sensitive, allowing the use of very dilute solutions and extending the range of substrate concentrations.

3. Automatic Titration (pH-Stat)

Used when a reaction produces or consumes an acid or a base. The instrument maintains constant pH and records the acid/base added as a measure of reaction progress.

4. Radioactivity Assays

Highly sensitive methods are used when substrates are labeled with radioactive isotopes.

Examples:

1- Tracking radioactive ATP using filtration and scintillation counting

2- Measuring peptide bond cleavage or protein synthesis using radiolabeled amino acids

Analysis of Very Fast Reactions

Some enzymatic processes occur too rapidly to be measured under steady-state conditions. Two major techniques are used:

1. Stopped-Flow Method
2. Temperature Jump (T-Jump)

1- Stopped-Flow Technique

The stopped-flow technique, shown in **Figure 11A.2**, is a powerful method for examining extremely rapid biochemical reactions. In this approach, the enzyme and substrate are kept in separate syringes and mixed within a few milliseconds as they pass through a rapid mixer.

The flow is then stopped suddenly in a stopping syringe, which simultaneously triggers detection—typically through **absorbance or fluorescence**. Because the detector is positioned only about 1 cm from the mixing chamber and the flow rate can reach up to 1000 cm/s, the reaction observed is approximately **1–5 milliseconds** old, corresponding to the instrument's **dead time (**The dead time is the short interval between mixing and detection during which the reaction cannot be observed). This allows researchers to monitor very fast events, including the initial enzyme–substrate encounter, early catalytic steps, and rapid binding or release processes.

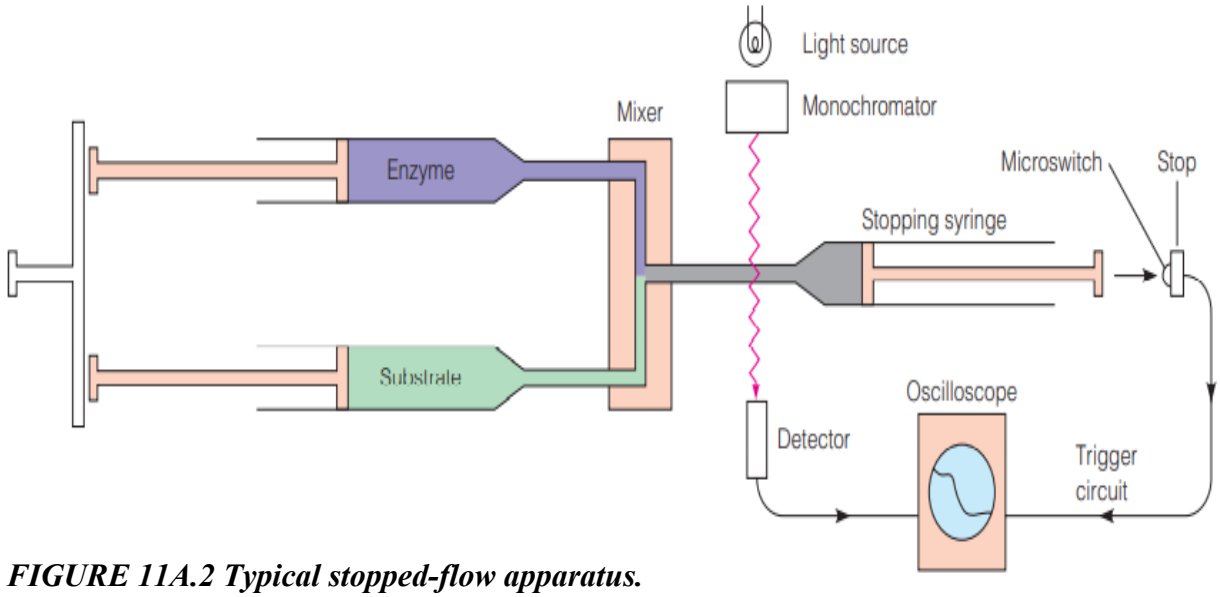


FIGURE 11A.2 Typical stopped-flow apparatus.

2- Temperature Jump (T-Jump) Technique

The temperature-jump method, shown in **Figure 11A.3**, is used to study reactions that are too fast to be captured by stopped-flow. In this technique, a reaction mixture at equilibrium is rapidly heated—usually by **5–10 °C** using a strong electrical pulse or a pulsed infrared laser. This sudden rise in temperature disrupts the existing equilibrium and forces the system toward a new one. The subsequent relaxation back to equilibrium is monitored by absorbance or fluorescence, allowing detection of processes on the **nanosecond to microsecond** timescale.

These features make T-jump particularly valuable for examining ultrafast conformational changes and the earliest pre-steady-state steps in enzyme catalysis.

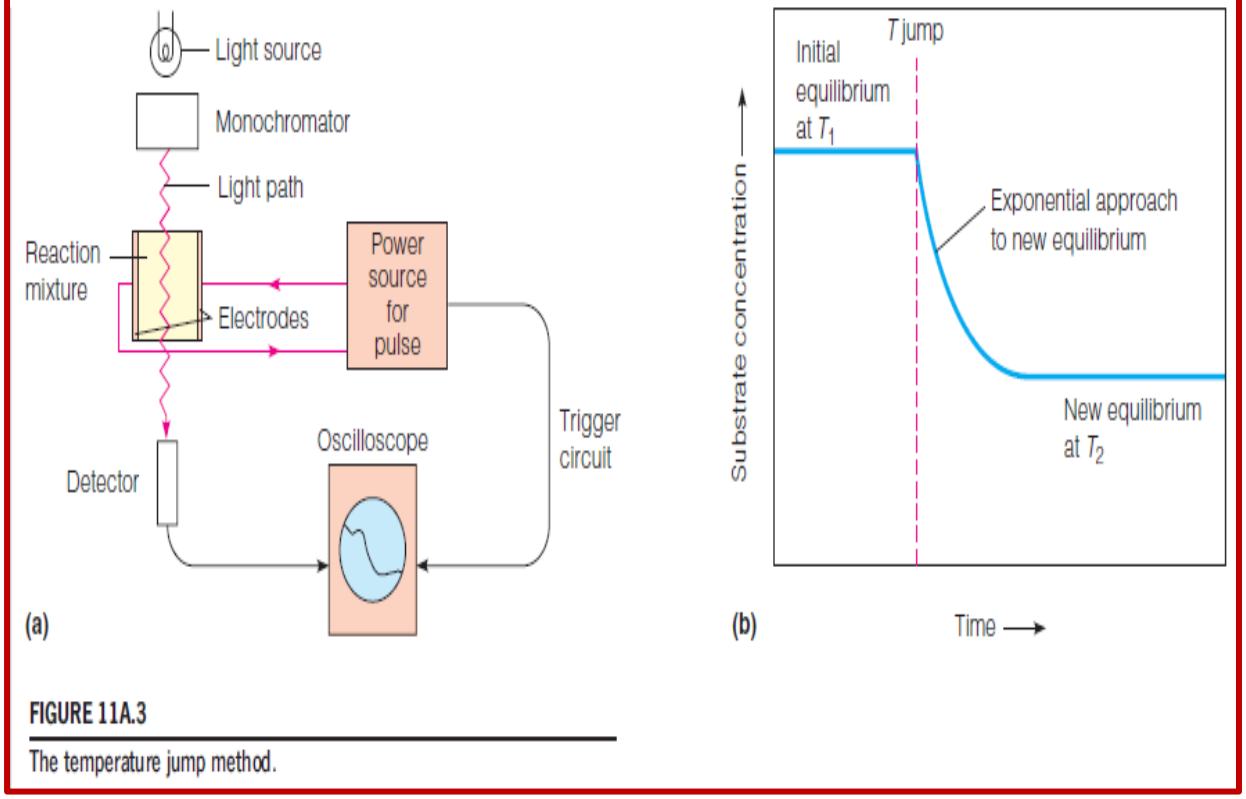


FIGURE 11A.3
The temperature jump method.