TOOLS OF BIOCHEMISTRY

How to measure the rates of enzyme-catalyzed reactions?

There are essentially two approaches to enzyme kinetic analysis. The first and simplest is to make measurements of rates under conditions in which the steady-state approximation holds (see page 432). Under these conditions, the Michaelis–Menten equation is often applicable, and determination of the reaction velocity as a function of substrate and enzyme concentrations will yield Almost all enzymatic studies at least start in this way. However if the experimenter wishes to learn more of the details of the mechanism, it is often important to carry out studies before the steady state has been attained. Such pre-steady-state experiments require the use of special fast techniques. On pages 437–438, we described how a combination of such approaches can be used to dissect a complex enzymatic process and to understand it in detail. Here we describe some of the experimental techniques that can be employed.

Analysis of the Steady State

The steady state in most enzymatic reactions is established within seconds or a few minutes and persists for many minutes or even hours thereafter.

Therefore, extreme rapidity of measurement is not important, and many techniques are available to the experimenter wishing to follow the reaction. Descriptions of the most commonly used techniques follow.

Spectrophotometry

Spectrophotometric methods are simple and accurate (see Tools of Biochemistry 6A). However, an obvious requirement is that either a substrate or a product of the reaction must absorb light in a spectral region where other substrates or products do not.

Dr. Zahraa M. A. Hmodat

Classic examples are reactions that generate or consume NADH. NADH absorbs quite strongly at 340 nm but does not absorb in this region. Thus we could, for example, follow the oxidation of ethanol to acetaldehyde, as catalyzed by alcohol dehydrogenase, by measuring the formation of NADH spectrophotometrically.

Fluorescence

The applications of fluorescence are similar to those of spectrophotometry, and the problems are similar:

A substrate or a product must have a distinctive fluorescence emission spectrum (see Tools of Biochemistry 6A).

However, fluorescence often has the advantage of high sensitivity, so extremely dilute solutions may be employed, enabling an experimenter to greatly extend the concentration range (i.e., [S]) over which studies are practicable.

Automatic Titration

If the reaction produces or consumes acid or base, it can be followed by using a device called a pH-stat. A glass elects the pH of the solution, and its signal is used to actuate a motor-driven syringe that titrates acid or base into the reaction vessel to keep the pH constant. The time-based record of acid or base consumed is then a record of the progress of the enzymatically catalyzed reaction.

Radioactivity Assays

If a substrate is labeled with a radioactive isotope that will be lost or transferred during the reaction to be studied, measurement of changes in radioactivity can be an extremely sensitive kinetic method. This procedure

Dr. Zahraa M. A. Hmodat

requires that the labeled compound can be separated quickly at different, precisely defined times during the reaction.

An example is a method often used with radioactive ATP. The ATP can be adsorbed on charcoal-impregnated filter disks by very fast filtration of aliquots from the reaction mixture. The radioactivity can then be measured in a scintillation counter (see Tools of Biochemistry 12A).

Another example of the use of radioisotopes comes from measuring the rates of peptide-bond cleavage (by a protease), or protein biosynthesis (e.g., ribosomal protein synthesis). Peptides are most commonly labeled with radioactive amino acids that contain or

The rate of a peptide cleavage or synthesis reaction can be monitored by rapidly precipitating the peptide (or peptide fragments) from the reaction solution using cold trichloroacetic acid and collecting the precipitate on filter paper. As described above, the radioactivity present on the filter paper can be quantitated using a scintillation counter

Analysis of Very Fast Reactions Reactions

Reactions that are extremely rapid require special techniques to investigate the pre-steady-state processes. Two major methods are currently employed to cover the rapid time scales shown in Figure 11A.1.

Stopped Flow

Figure 11A.2 shows a stopped-flow apparatus, first described by Quentin Gibson in the 1950s. Enzyme and substrate are initially in separate syringes. The syringes are driven, within a few milliseconds, to deliver their contents through a mixing chamber and into a third, “stopping” syringe. This step triggers a detector

Dr. Zahraa M. A. Hmodat

to begin observing (for example, by light absorption or fluorometry) the solution in the tube connecting the mixer to the stopping syringe.

Flow rates can easily be made as high as 1000 cm/s. If the mixture was moving at this rate when the flow was stopped, and if the observation point is 1 cm from the mixer, the detection system first sees a mixture that is 1 ms "old." The reaction can then be followed for as long as desired—often for a period of only a few seconds. The limitations of the method are imposed only by the initial "dead time" (i.e., the time it takes the mixed solutionsto arrive at the detector—in the example above, 1 ms) and the rapidity of the detection system.

Stopped-flow is used to measure rates of rapid enzymatic reactions as well as ligand binding events, such as binding to, or release from, hemoglobin (see Chapter 7)

A. Hmodat

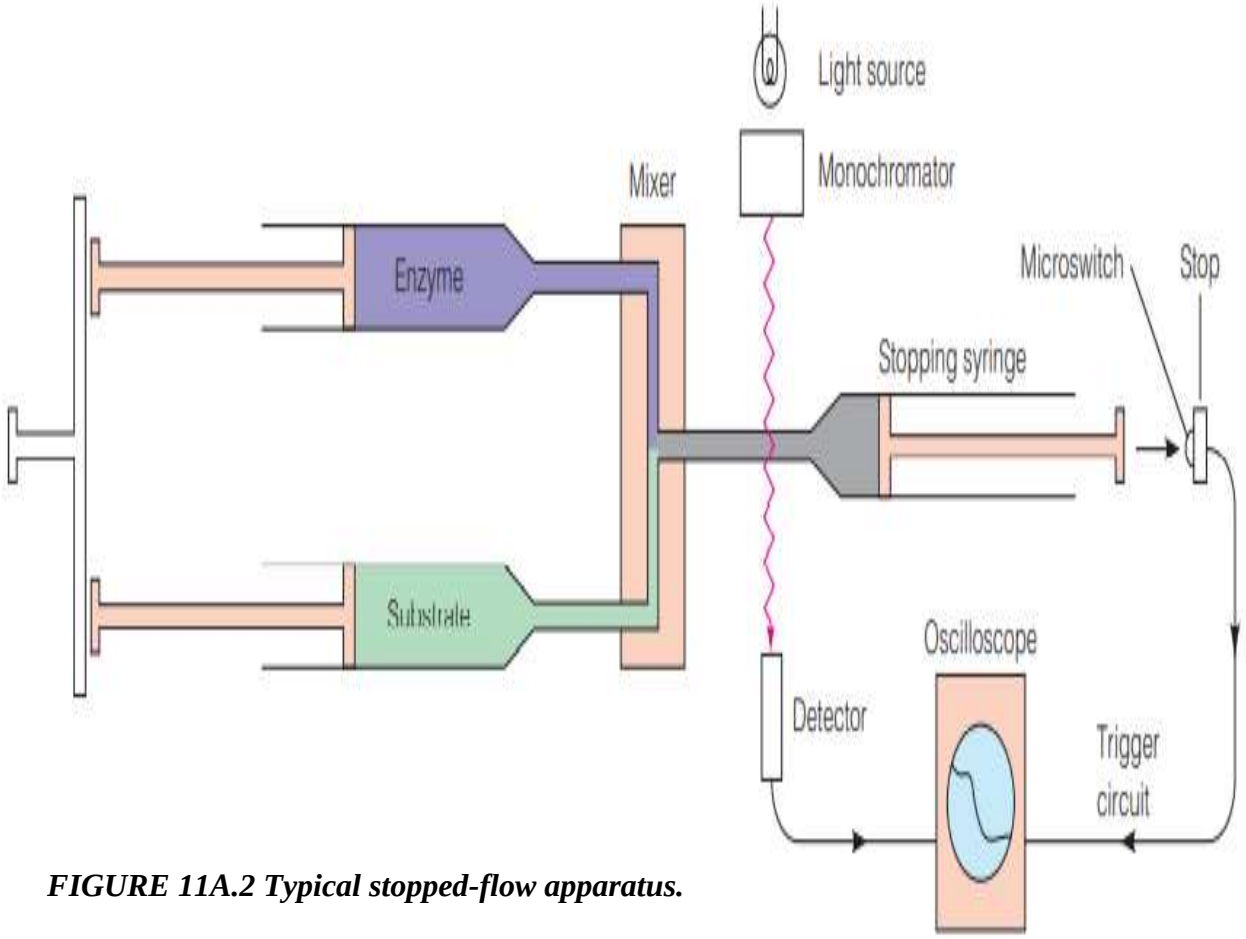


FIGURE 11A.2 Typical stopped-flow apparatus.

Temperature Jump

Some processes are so fast that they are essentially completed in the dead time of a stopped-flow apparatus. The experimenter may then turn to temperature jump (T-jump) methods. The basic apparatus and principle of the method are shown in Figure 11A.3a and b, respectively. A reaction mixture that is at equilibrium at a temperature is suddenly jumped to a temperature Because chemical equilibria are typically temperature-dependent, the position of equilibrium will shift, and the system must now react to attain this new equilibrium. A rapid jump in temperature (5–10 °C in) can be obtained by passing a large burst of electrical current between electrodes immersed in the reaction mixture. Even more rapid jumps (10–100 ns) can be obtained if a pulsed infrared laser is used to heat the mixture. The relaxation (approach) to anew equilibrium, monitored by absorption or fluorescence measurements, is an exponential process. For a simple reaction, the change in reactant concentration is given by

$$\Delta[A] = (\Delta[A]_{total})e^{-t/\tau} \qquad (11.A1)$$

Where ***T*** is called the relaxation time and can be related to the rate constants for the reaction. For example, for the simple reversible reaction

$$A \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} B$$

we have

$$\frac{1}{\tau} = k_1 + k_{-1} \qquad (11.A2)$$

More complex reactions involve multiple relaxation times and more complex curves than expressed by equation (11A.1). ***T*** -jump experiments are appropriate for reactions with ***T*** values as low as about Although a number of other techniques are employed for even faster reactions, including some newly developed NMR 10^{-5} s.

Although a number of other techniques are employed for even faster reactions, including some newly developed NMR methods and pulsed laser techniques, the methods described here are widely used. If we consider the variety of techniques available to the experimenter, we can see that they cover a wide time range. Altogether, times from nanoseconds to hours can be studied.

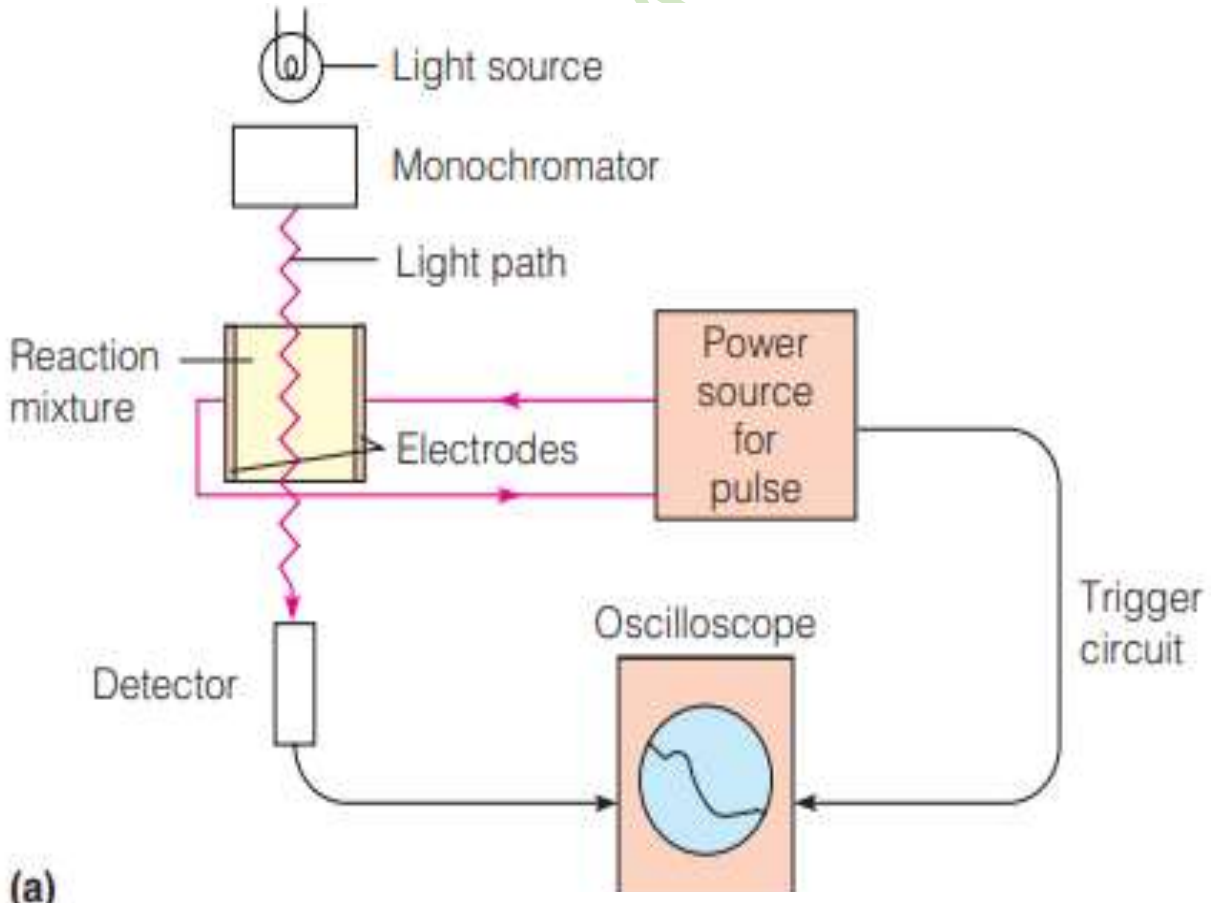


FIGURE 11A.3 The temperature jump method

The Diversity of Enzymatic Function Classification of Protein Enzymes

By this point, it should be clear that an enormous number of different proteins act as enzymes. Many of these enzymes were given common names, especially during the earlier years of enzymology.

Some enzyme names, like triose phosphate isomerase, are descriptive of the enzyme's function; others, like trypsin, are not.

To reduce confusion, a rational naming and numbering system has been devised by the Enzyme Commission of the International Union of Biochemistry and Molecular Biology (IUBMB).

Enzymes are divided into six major classes, with subgroups and sub-subgroups to define their functions more precisely. The major classes are as follows:

1- **Oxidoreductases catalyze** oxidation–reduction reactions.

2- **Transferases catalyze** the transfer of functional groups from one molecule to another.

3- **Hydrolases catalyze** hydrolytic cleavage.

4- **Lyases catalyze** the removal of a group from or the addition of a group to a double bond, or other cleavages involving electron rearrangement.

5- **Isomerases catalyze** intramolecular rearrangement.

6- **Ligases catalyze** reactions in which two molecules are joined.

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

Dr. Zahraa M. A. Hmodat

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.

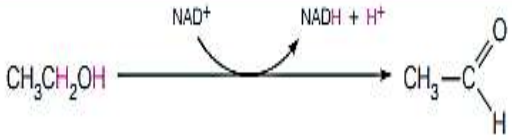
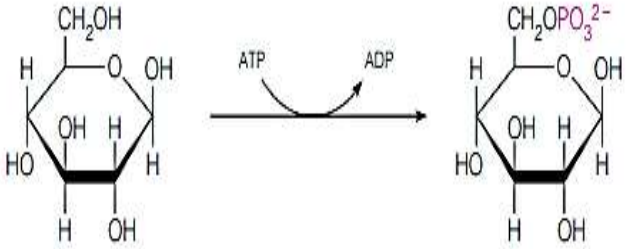
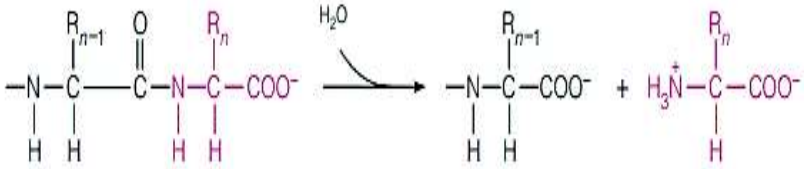
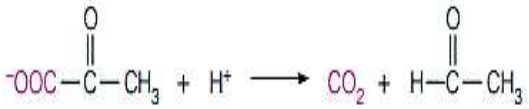
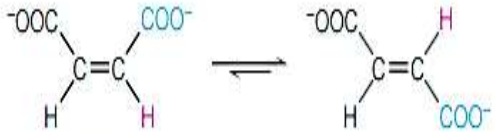
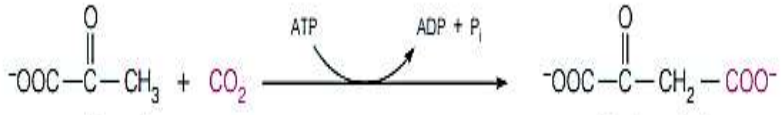
Listings for almost all currently recognized enzymes, together with information on each enzyme and literature references, can be found in online databases such as BRENDA (Braunschweig ENzyme DAtabase; http://www.brenda-enzymes.info) or ExPASy (Expert Protein Analysis System; http://expasy.org/enzyme). The 5000+ entries in these databases do not include all enzymes; more are being discovered all the time.

Indeed, it has been estimated that the typical cell contains many thousands of different kinds of enzymes.

Table 11.10 lists one example enzyme and reaction from each of the major classes. We will discuss each of these reactions later in this book. The main point here is the enormous diversity of enzymatic functions and how their nomenclature has been rationalized.

Dr. Zahraa M. A Hmodat

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^+)	 Ethanol — Acetaldehyde
2. Transferases	Hexokinase (EC 2.7.1.2) (phosphorylation)	 D-Glucose — D-Glucose-6-phosphate
3. Hydrolases	Carboxypeptidase A (EC 3.4.17.1) (peptide bond cleavage)	 C-terminus of polypeptide — Shortened polypeptide — C-terminal residue
4. Lyases	Pyruvate decarboxylase (EC 4.1.1.1) (decarboxylation)	 Pyruvate — Acetaldehyde
5. Isomerases	Maleate isomerase (EC 5.2.1.1) (*cis–trans* isomerization)	 Maleate — Fumarate
6. Ligases	Pyruvate carboxylase (EC 6.4.1.1) (carboxylation)	 Pyruvate — Oxaloacetate

Molecular Engineering of New and Modified Enzymes

NOTE: 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.

Despite the variety of enzymatic functions available in nature, modern biotechnology continually faces the need for substances with new catalytic abilities, or enzymes that function with different specificities or under unusual conditions.

These needs have generated a field of enzyme design and engineering, which has enormous potential in the design of industrial catalysts as well as biopharmaceuticals.

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

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.

Dr. Zahraa M. A. Hmodat