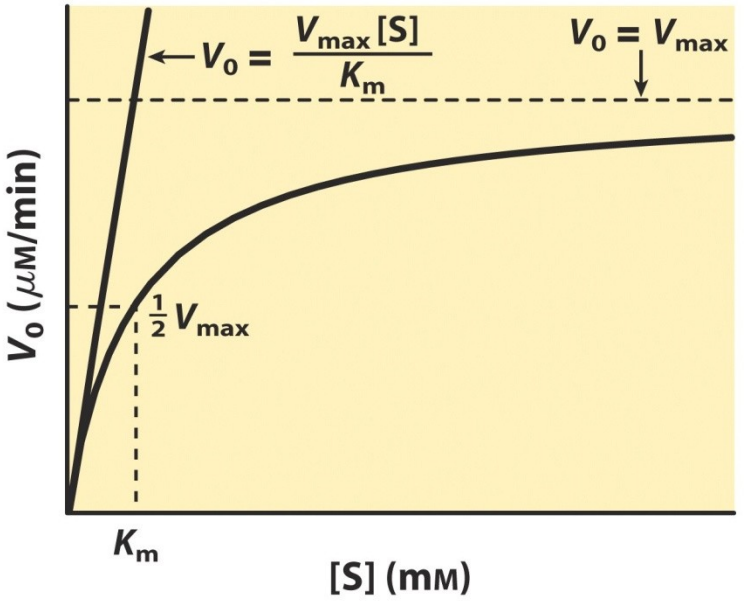
3. Analysis: Measurement of Protein and Enzyme Activity

3.1 Basic Principles

Michaelis Constant : Km

- **Michaelis-Menten equation**
  - $V_o = V_{max}\,[S] / (K_m + [S])$
- **When Vo = ½ V_{max}**
  - ½ = [S] / (K_m + [S])
  - K_m = [S]
- ***K_m***
  - Substrate concentration at V_o = ½ V_{max}



Substrate Concentration, Activators, and Inhibitors

- **Enzyme obeying Michaelis-Menten kinetics**
  - Use substrate concentration at least 10 x Km
    - 91% of Vm
    - Little difference from slight variation between assays
    - Consumption of substrate during the assay makes little difference to the rate
    - Less inhibitory effect by the products

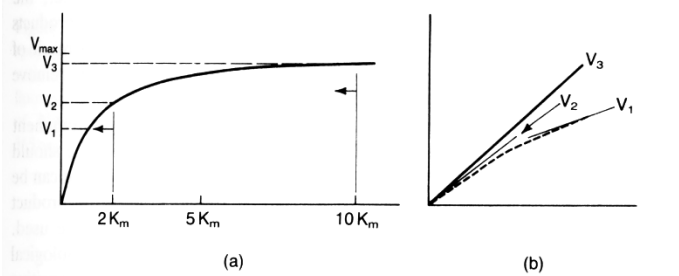


Figure 3.1. Effect of substrate consumption on enzyme activity: (a) relationship between rate and substrate concentration; (b) observed rates as substrate is consumed, starting with [substrate] = $10K_m$ (*solid line*) and [substrate] = $2K_m$ (*dashed line*).

  - Sequential mechanism using two subtrates
    - Higher concentration of A → lower Km for B
  - Nonsequential (Ping-Pong) mechanism
    - Higher concentration of A → Higher Km for B

Production Inhibition

- **Overcome problems of product inhibition and approach to equilibrium**
  - Use very high concentrations of substrate
  - Use unphysiological pH (if a proton is involved in the reaction)
  - Use very sensitive method for detecting product

- **Malate dehydrogenase**
  - $Malate^{2-} + NAD^{+} \leftrightarrow oxaloacetate^{2-} + NADH + H^{+}$
  - Reverse reaction at pH7 (V_{max})
    - Detection of the loss of NADH : loss of absorbance at 340 nm
  - Force the reaction to go in the forward direction (1/2 V_{max})
    - High concentration of malate (50-100 mM)
    - High pH to remove protons
    - Removal of oxaloacetate by trapping as a hydrazone

Substrate Inhibition

- **Inhibition of enzyme activity at high substrate concentration**
  - Reaction of substrate with another substrate
  - Increase in ionic strength by ionic substrate
  - Substrate includes an inhibitor contaminant

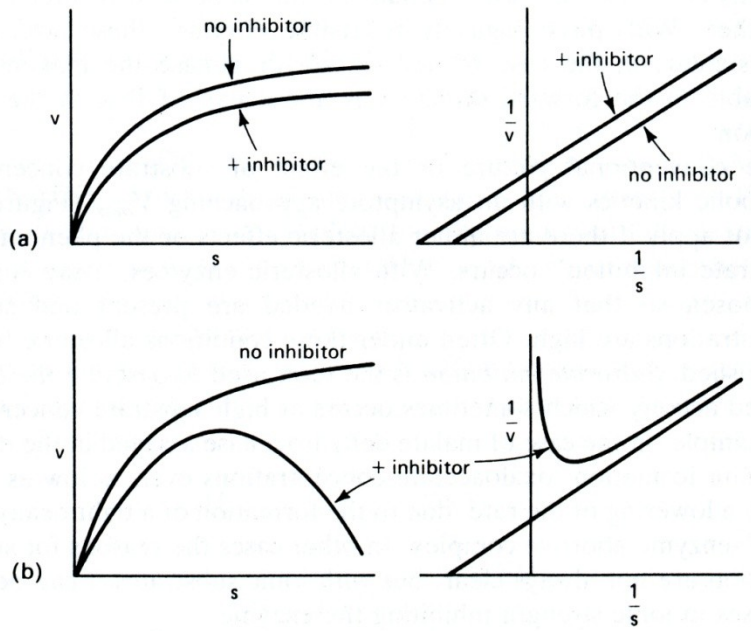


Figure 3.3. (a) The effect of a competitive inhibitor present in the substrate preparation; rate versus substrate, and reciprocal plots; (b) as (a), but with a noncompetitive inhibitor in the substrate.

pH, Ionic Strength, and Temperature

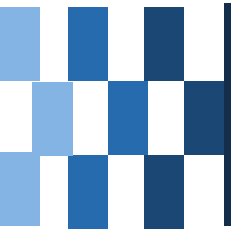
- **K_m and k_{cat} is dependent on**
  - pH, ionic strength, and temperature
- **Optimum pH for an enzyme**
  - Obtained with high substrate concentration
  - Can be different from physiological pH optimum
  - Alkaline phosphatase
    - pH optimum 10-11
    - Naturally operate at pH7 : lower K_m than at pH10

Establishment of Enzyme Assay 1

- **High substrate concentration**
- **Optimum pH for optimal value of v**
- **Proper buffer**
  - Phosphate or multiple charged anionic buffers (10~100 mM)
    - Can compete with negatively charged substrates
  - Salts
    - Can be essential for enzymes from thermophiles or halophiles
  - Essential metal ions
    - Can be complexed by buffers (citrate and histidine)
  - Ionic strength and salts
    - Can affect enzyme activity
    - Physiological ionic strength: 0.1-0.2 M
    - Salt concentration higher than 0.2 M : often depress activity

Establishment of Enzyme Assay 2

- **Optimum temperature**
  - Not a fundamental property
    - Depending on the incubation time
      - Increase in enzyme activity by a factor 1.5 ~2.5 for every 10 ^{o}C
      - Denaturation at high temperature
    - The shorter the incubation time the higher will be the apparent optimum T
    - Typically 40-60^{o}C for 5-10 min reaction
  - Q10
    - Activity at T + 10 ^{o}C / activity at T



3.2 Stopped Methods

Stopped Method

- **Method**
  - Incubation of the enzyme with its substrates for a fixed period of time
  - Stop the reaction and measure the products
- **Factors to be considered**
  - Check the linearity
    - Slowing down after long incubation
    - Lag in the beginning
- **Stopping methods**
  - Instantaneous denaturation of the enzyme
    - Acidification (3-5% Trichloroacetiate or perchlorate ppt.) and neutralization with KOH containing some K_2CO_3
    - Rapid heating
    - 1% SDS : protein denaturation
  - Inactivation of the enzyme
    - pH change
    - Chelating metal ions with EDTA
    - Adding cold substrate in radiochemical assay
    - Add inhibitors

Measurement of Product

- **Methods**
  - Enzymatic
  - Chemical
  - Radiochemical
- **Enzymatic methods**
  - Enzymatic conversion of the product to an observable compound
  - Detection by spectrophotometry, fluorometry, bioluminescence, gas production
  - e.g. Fructose 1,6-biosphosphatase
    - Enzyme reaction: Fru1,6 BP → Fru6P + Pi
    - Detection with coupling enzymes
      - E_P: Fru6P → Glc6P (phosphoglucose isomerase)
      - E_Q: Glu6p + NAD+ → 6P-gluconate + NADH (Glc6P dehydrogenase)
      - NADH detection at 340 nm

Enzymatic Detection of the Product

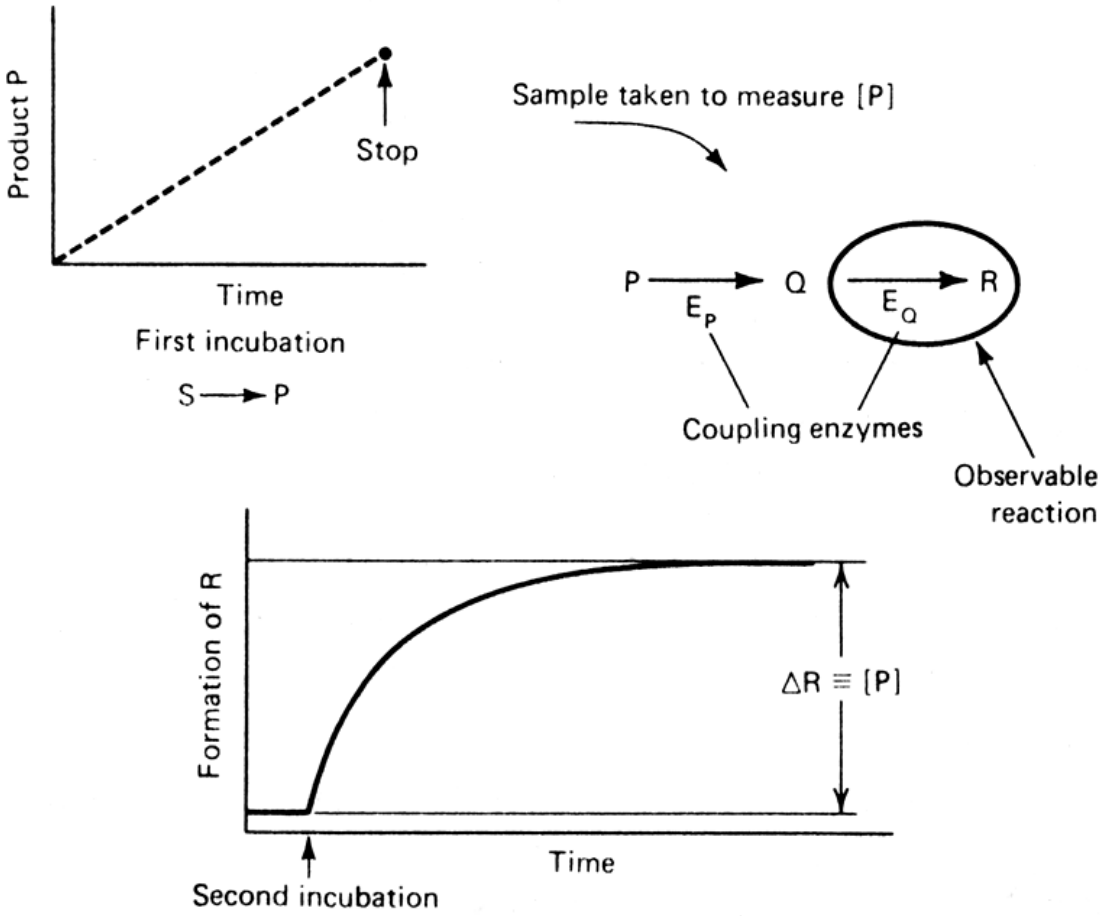
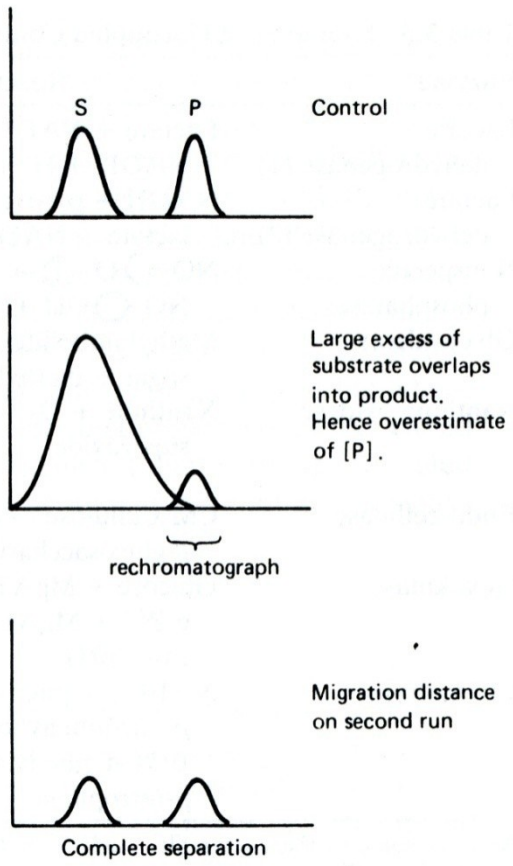


Figure 3.5. Scheme for measuring enzyme activity, using a stopped method followed by enzymatic detection of the products.

Measurement of Product

- **Radiochemical methods**
  - Detection of the labeled products separated from the labeled substrate
  - Separation of the product
    - Phase separation
    - Chromatography: TLC, ion exchange, GPC
      - – Possible problem in separation
- **HPLC**



3.3 Continuous Methods

Continuous Methods

- **Continuous methods**
  - Continuous detection of product formation
- **Types**
  - Uncoupled method
    - Direct detection of the products
      - UV observance
      - Fluorescence
      - Change in viscosity
      - Enthalpy change detected by calorimeter
  - Coupled method
    - Chemical or enzymatic conversion of a product to an observable form
    - Requirements for the added reagents
      - Must work in the same conditions as the enzyme being measured
      - Must not interfere with the enzyme's activity

Uncoupled method

Table 3.3. Examples of Uncoupled Continuous Assays

Enzyme	Reaction	Observation
Lactate dehydrogenase (1)	Lactate + NAD^+ → pyruvate + NADH + H^+ (high pH)	NADH formation at 340 nm
Lactate dehydrogenase (2)	NADH + pyruvate + H^+ → lactate + NAD^+	NADH loss at 340 nm
Nonspecific phosphatases	NO_2⬡-O-Ⓟ → NO_2⬡-OH + P_i	Nitrophenol production at 400 nm
Glycosidases	Methylumbelliferyl-glucoside → sugar + methylumbelliferone	Fluorescence of methylumbelliferone
Xanthine oxidase	Xanthine + O_2 → uric acid + superoxide	Increase in absorbance at 295 nm due to uric acid
Endo-cellulase	CM-Cellulose → oligo β-glucosaccharides	Loss in viscosity
Hexokinase	Glucose + $MgATP^{2-}$ → glucose 6-P^{2-} + $MgADP^-$ + H^+ (high pH)	Acid production (pH-stat)
Chymotrypsin	*N-t*-BOC-L-phenylalanine *p*-nitrophenyl ester → *N-t*-BOC-L-phenylalanine + *p*-nitrophenol	Nitrophenol production at 400 nm

Note: In some of these examples stopped methods have greater sensitivity, since maximum absorption or fluorescence may be obtained at a pH outside the enzyme activity range.

Coupled Method

- **Enzymes involving acyl-CoA**
  - Acyl-S-CoA + HX → Acyl-X + HS-CoA
  - HS-CoA + DTNB → TNB-S-CoA + TNB
  - Detection of yellow TNB at 412 nm
- **Triose-P isomerase**
  - Glyceraldehyde 3-P → dihydroxyacetone-P
  - dihydroxyacetone-P + NADH → glycerol 1-P + NAD^+
  - Detection of NAD+ by decrease in observance at 340 nm
- **Detection of ADP**
  - Regeneration of ATP by pyruvate kinase
  - Detection of NAD^+ generated by lactase dehydrogenase

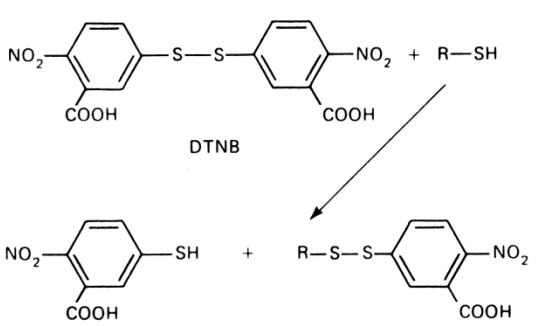


Figure 3.7. Reaction of DTNB with sulfhydryl compound to liberate colored product.

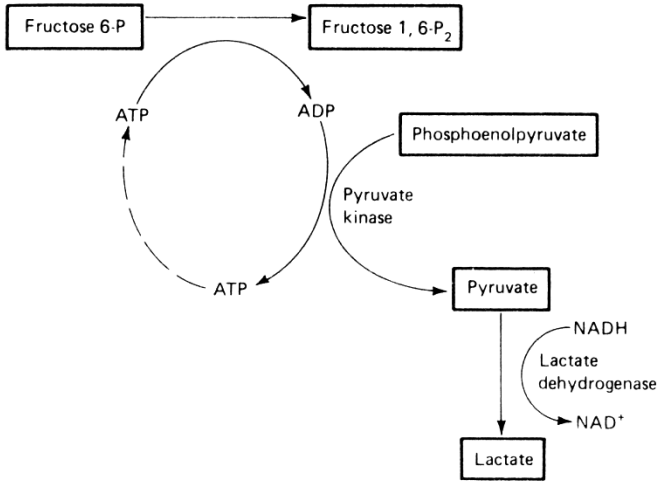
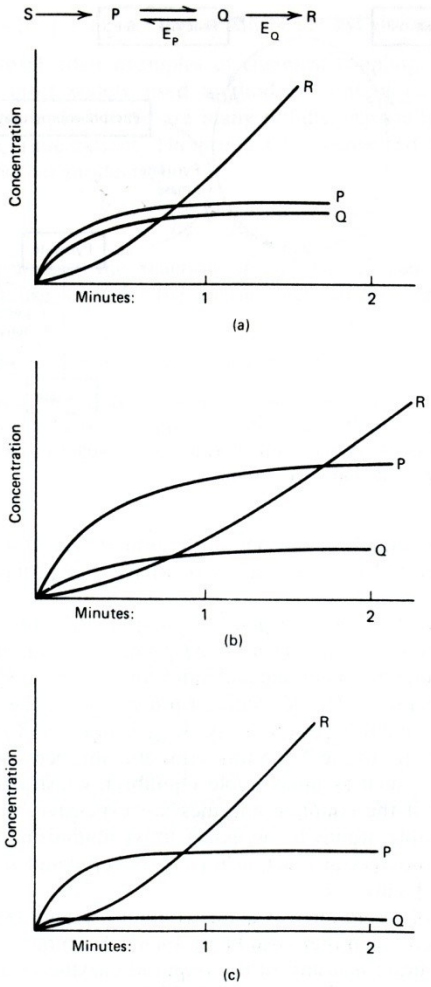


Figure 3.8. Principle of the coupled assay for phosphofructokinase using lactate dehydrogenase as the indicator enzyme.

Coupled Method

- **Amount of indicator enzyme**
  - V_{max}/K_m = 5 min^{-1} to reach a rate of at least 98% of the primary enzyme within 1 min



V_{max}/K_m = 5 min^{-1}

P→ Q irreversible

V_{max}/K_m = 5 min^{-1}

P→ Q reversible

V_{max}/K_m = 25 min^{-1}

P→ Q reversible

Figure 3.9. Two-step coupled reaction where product P is converted to Q and then to R by enzymes E_P, E_Q. Concentrations of P, Q, and R (a) when $V_{max}/K_m = 5\,min^{-1}$ for both E_P and E_Q, and $P \rightarrow Q$ is essentially irreversible. (b) $V_{max}/K_m = 5\,min^{-1}$ for both E_P and E_Q, and $P \rightarrow Q$ is reversible when equilibrium favoring P. Note that rate of formation of R is still increasing at 2 min. (c) as (b), but $V_{max}/K_m = 25\,min^{-1}$ for E_Q. Rate of formation of R reaches a steady state by 1 min, with $[Q]$ much lower than in (b).

Coupled Method

- **Correction of contaminating activities**
  - Use blank reaction

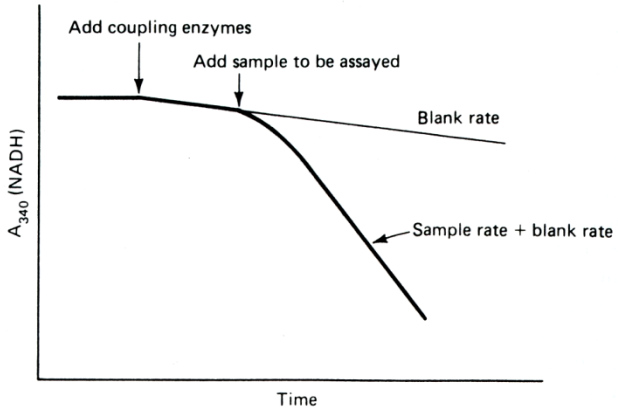


Figure 3.10. Progress of a coupled reaction involving oxidation of NADH, in which there is a blank rate due to contamination in the coupling enzymes.

- **Problems of assays with crude extract**
  - The crude extract may contain other substrates
  - Other enzymes in the extract may be interfering with the coupling system being used

Stopped vs. Continuous Enzyme Assay

Table 3.4. Comparison of Advantages and Disadvantages of Stopped and Continuous Enzyme Assays

Stopped assays	Continuous assays
No limitation on conditions	Conditions may be limited by requirement of coupling system
Very small volume saves on expensive reagents	Usually requires larger amounts of reagents, though microcells result in saving
High sensitivity for radiochemical methods	Sensitivity usually low compared with radiochemical methods
Single point determination may hide irregular activity	Continuous observation detects nonlinearity
Result not instantaneous	Instantaneous result
Multi-manipulation more time-consuming	Simple manipulation
No coupling enzymes required in assay (but may be needed to determine product)	Coupling enzymes needed may be expensive and/or contain contaminants
Product accumulation may cause inhibition[a]	Product may be removed by coupling system

[a] A nonobservable coupling system can be included to remove products.