Lecture #2: **Enzyme Purification and Assay**

(1) Initial velocity measurements
- **Effect of enzyme concentration**
- **Effect of temperature**

(2) Assay types
- **Discontinuous**
- **Continuous**
- **Quantitative assays**
- **Coupled reactions**

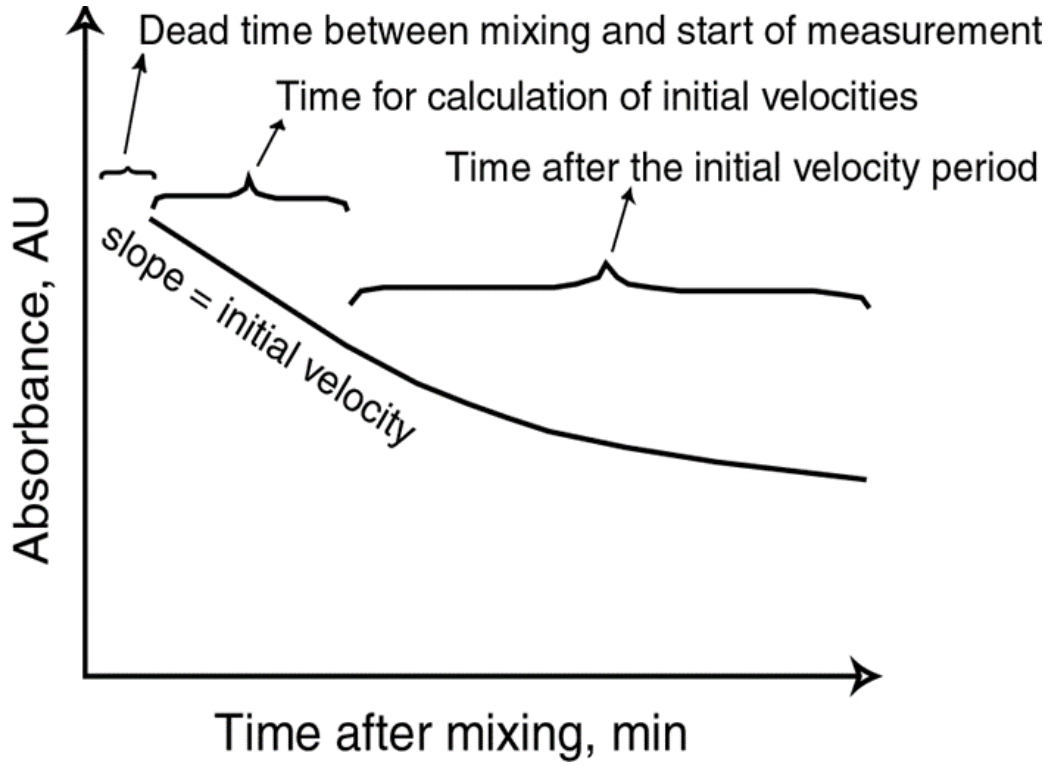
(3) Enzyme activity measurements
- **Units**
- **Katals**

(4) Turnover number and properties

(5) Purification and assessment
- **Purification table**

(6) Methods for measurements
- **Continuous systems**
- **Immobilized enzymes**
- **Stopped flow**
- **Quench flow**
- **Multi-plate readers**

How to Perform Kinetic Measurements



1. **Dead time**
   - time after mixing the sample and before the measurement begins
2. **Initial slope**
   - Linear portion of the time-course plot for data collection
3. **Post collection**
   - Time after initial velocity during which the reaction reaches equilibrium

1. Initial velocity measurements

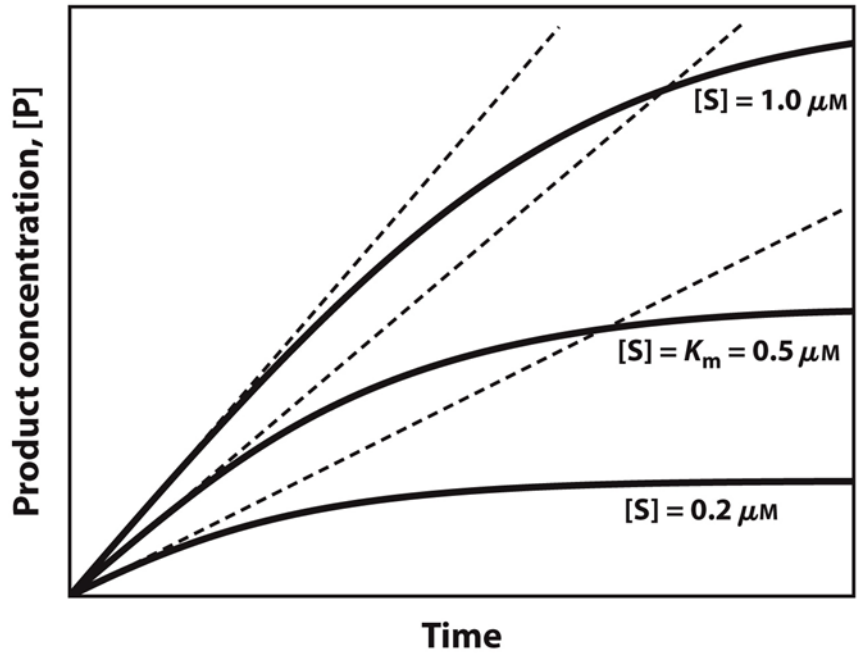


Figure 6-10
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• a typical enzyme reaction is reduced as the reaction proceeds <u>because:</u>

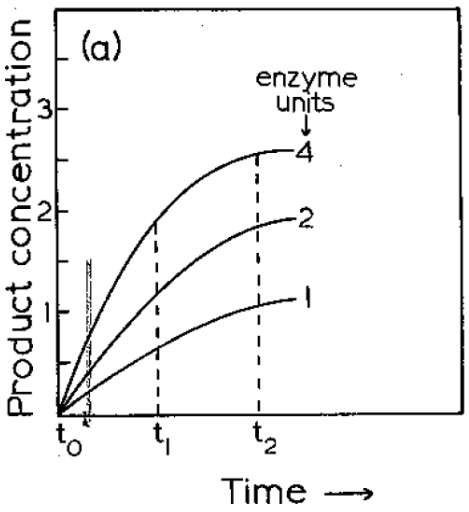
i. denaturation of enzyme
ii. product inhibition
iii. decrease of enzyme saturation
iv. inactivation of coenzyme
v. increase of reverse reaction

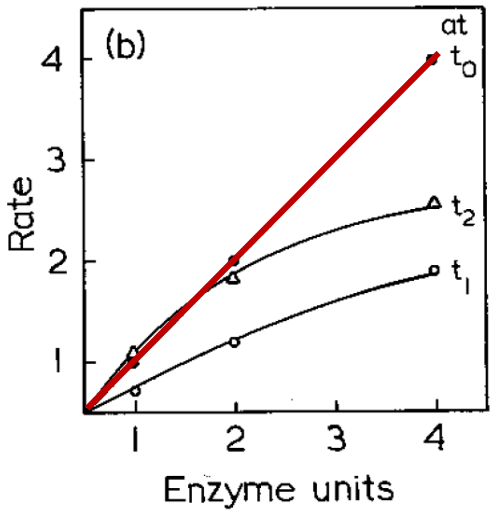
•critical that **velocity be determined at the <u>very beginning</u> of the reaction**

•initial velocity is the slope of the curve depicting S or P changes with time

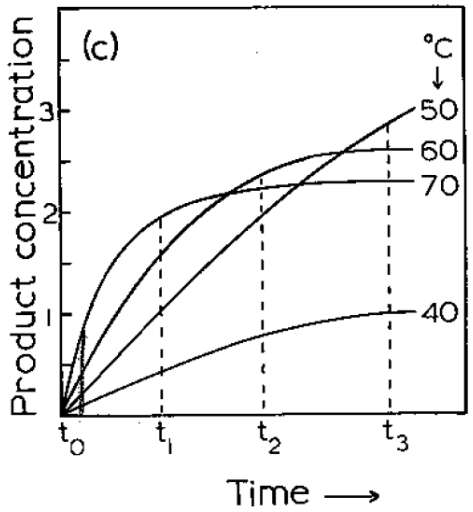
$$v_o = d[P]/dt = -d[S]/dt$$

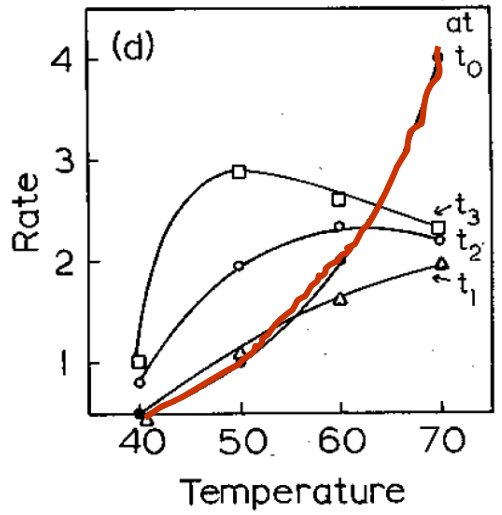
•in practice: $v_o = \Delta[P]/\Delta t = -\Delta[S]/\Delta t$ **<u>provided that</u>** these parameters are measured at the **<u>beginning</u>** of the **reaction** where the rate of S consumption and P formation is linear with time





- By choosing an arbitrary incubation time, the incubation time itself now becomes a variable
- Can be avoided by measuring initial velocities





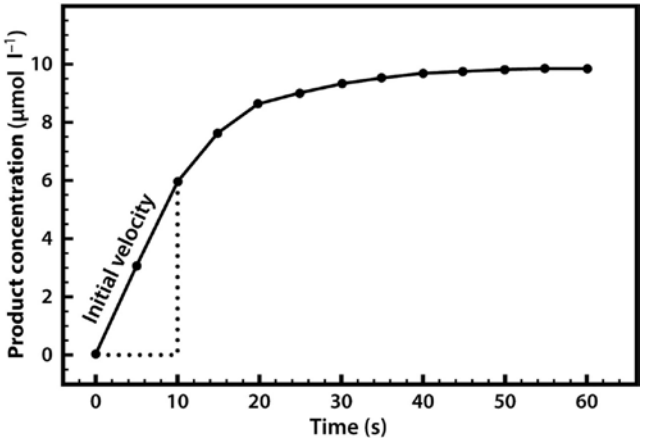
Core Topics in Biochem., Stenesh (1993)

2. Assay types

i. Discontinuous assay - fixed time point sampling of kinetic data

- Radiometric: measure incorporation of radioactivity into substrates
- Chromatographic: measure product formation by separating the reaction mixture into components by chromatography (HPLC, FPLC, TLC)

•the rate of [P] formation must be linear over the incubation time chosen

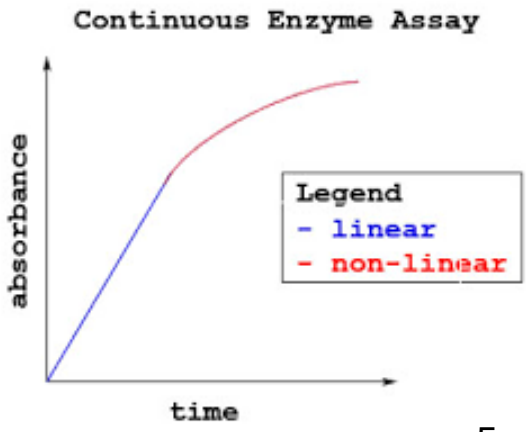


ii. Continuous assay - reaction is analyzed continuously using a monitoring technique

- Spectrophotometric, calorimetric, chemiluminescent

•for the continuous assay one must use the initial part of the curve for dP/dt

•one must use [E] that falls within the range in which P formation varies linearly with [E] for all substrate concentrations



Two types of quantitative enzyme assays

(1) Measure the amount of E in the sample

- E is present at **much lower** concentration than S
- E is present in **limiting amounts** and S is present **in excess**
- assay conditions correspond to levelled off portions of v_o versus [S] curves

thus velocity α [E]

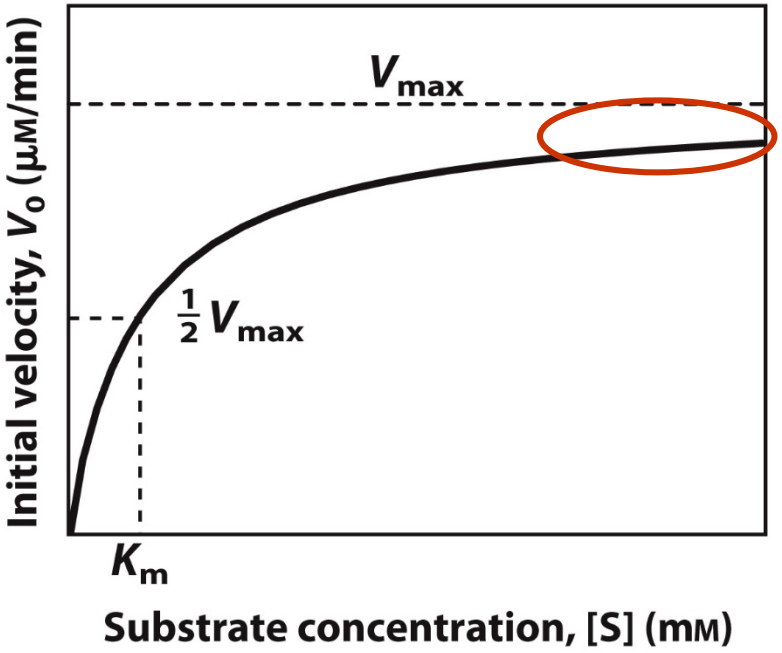


Figure 6-11
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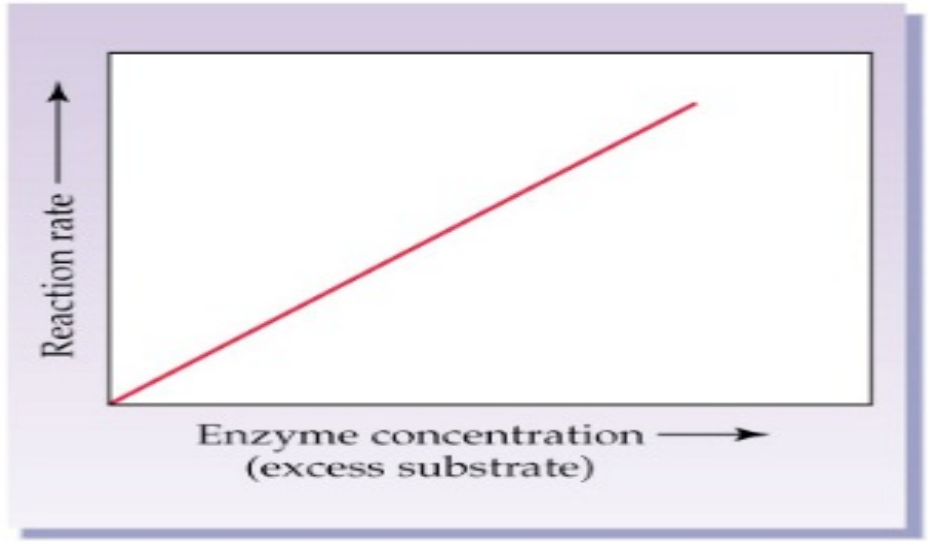


Zero order reaction

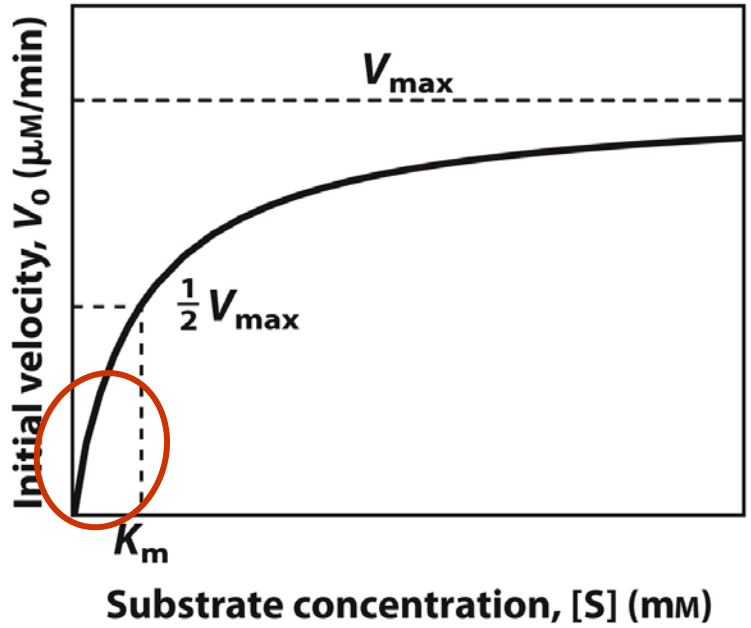
$V_0 = [E] * k * [S]^0$

(2) Measure the amount of S present ([S])

•[E] is high enough to convert all S $\rightarrow$ P

•S is limiting reagent

•assay conditions correspond to those of initial part of v_o versus [S] curves



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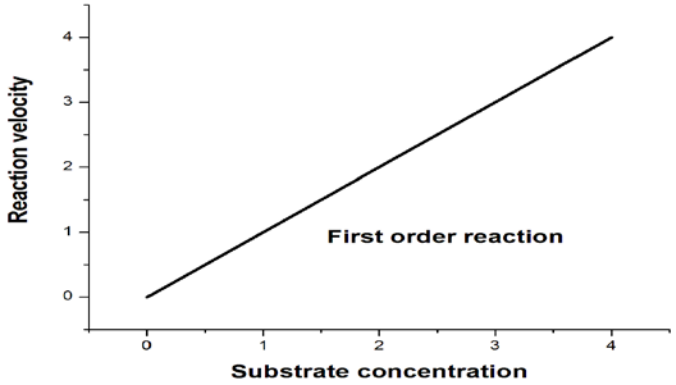


$V_0 = [E] * k * [S]^1$

First order reaction

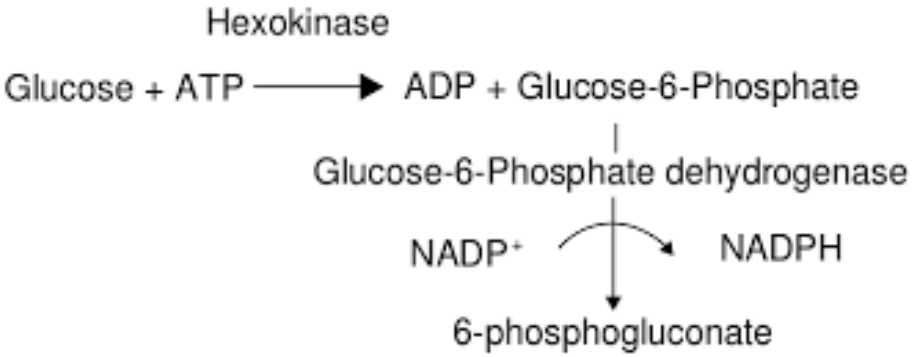
(3) Coupled Reactions

- some reactions difficult to assay because of **absence of a readily measurable** compound or property

- couple one reaction to another which produces a measurable product

$$A \xrightarrow{E_1} B \qquad \text{Reaction 1}$$

$$B \xrightarrow{E_2} C \qquad \text{Reaction 2}$$



- if conditions for E_2 are compatible with those of E_1 then both stages can be measured **simultaneously**
- <u>if not</u>, then reaction with E_1 is run first and stopped (boiling, change pH, etc.)
- second reaction (with E_2 in excess) is added and reaction proceeds until all of B is converted to C
- for such "coupled reactions" E_2 must be in **sufficient concentration** so that all of B is converted to C
    - Rate-limiting step in the linked assay is the **action of the first enzyme**

-the relative amounts of E_1 and A depend on the nature of assay

-if E_1 is being measured then A should be in **high concentration**

-if A is being measured then A should be in **low concentration**

3. Enzyme units of activity

- enzyme solutions described in terms of arbitrary units because enzymes are **active at very low concentrations** and purity is frequently unknown

Unit (definition)

- one international unit = amount of enzyme catalyzing the transformation of **1 µmol of substrate per min** under defined conditions
    - based on measurements of **initial velocity**

-greater the **purity** of an enzyme, the greater the fraction of total protein that is ***actually enzyme protein***

-relationship defined as "**specific activity**"

-**specific activity** - number of enzyme units per amount (mg) of total protein= **U/mg protein**

-**total activity** = U/mg protein x total mg protein
= U/mL of fraction x total vol (mL) of fraction

I.U.B. proposed **katal** (1972) ≡ SI Unit of activity

katal is amount of enzymatic activity that catalyzes the transformation of **1 mole of substrate per second** under defined conditions

$1\ U = 10^{-6}$ moles/60s = 16.7×10^{-9} mol/s
- 1 U = 16.7 nkatal
- 1 nkatal = 0.06 U
-1 katal = 6×10^{7} U

-concentration of enzyme in terms of katals is **"molar activity"**

-defined as the number of *katals per mole of enzyme*

4. Turnover number (TN, k_{cat})

-number of moles of substrate transformed into product per unit time per mole of enzyme under optimal conditions

-**molecular activity** = TN (per mole of enzyme)

-**catalytic centre activity** = TN (per mole of active sites)

•TN = $V_{max}/[E_T]$ = μmol(S→P) x min^{-1} x mL^{-1}/ (μmol enzyme or cat site x mL^{-1})

= min^{-1} or $\mathbf{t^{-1}}$

-typical values fall in the range of 50 - 10^7 min^{-1}

TABLE 6–7 Turnover Number, k_{cat}, of Some Enzymes

Enzyme	Substrate	k_{cat} (s^{-1})
Catalase	H_2O_2	40,000,000
Carbonic anhydrase	HCO_3^-	400,000
Acetylcholinesterase	Acetylcholine	14,000
β-Lactamase	Benzylpenicillin	2,000
Fumarase	Fumarate	800
RecA protein (an ATPase)	ATP	0.5

Table 6-7
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5. Purification and assessment

-purification is the process by which an enzyme is separated from other cell components

Three categories of purification methods:

i. **Precipitation** ⇒ differential solubility

- salting out with $(NH_4)_2SO_4$ most common.
- salts neutralize exterior charge of protein allowing aggregation.
- isoelectric precipitation uses same principle

ii. **Chromatography**

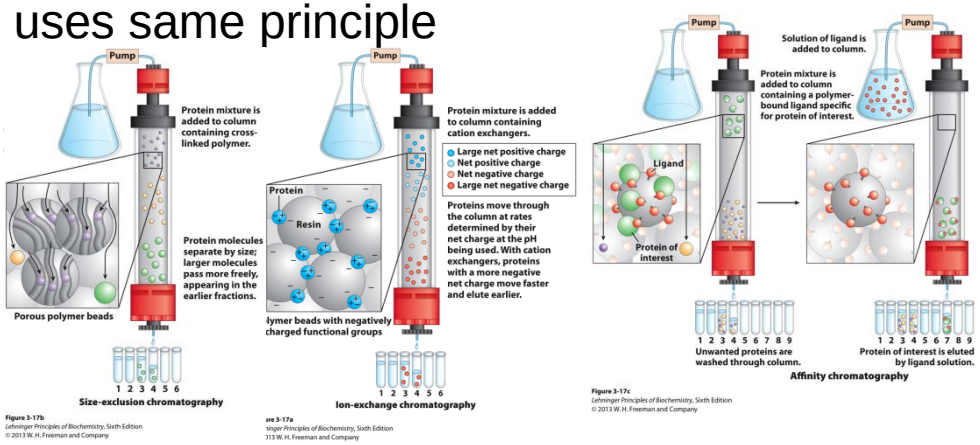
-various types, including

-gel filtration

-ion-exchange

-hydrophobic

-adsorption



-affinity: taken over with recombinant enzymes today!

-His tags, Flag tags, GST-tags

iii. **Electrophoresis**

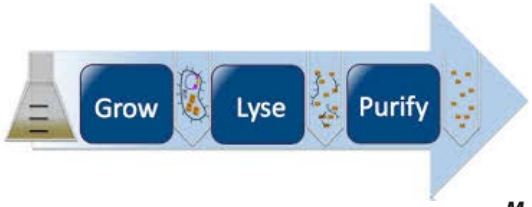
-native or denaturing

-presentation of purification data is vital for analysis of purity and recovery

-aim is to calculate **yield** and **purification factor**

- need 3 measurements:

i. volume of sample (mL)
ii. protein content (mg/mL)
iii. enzyme activity (units/mL)



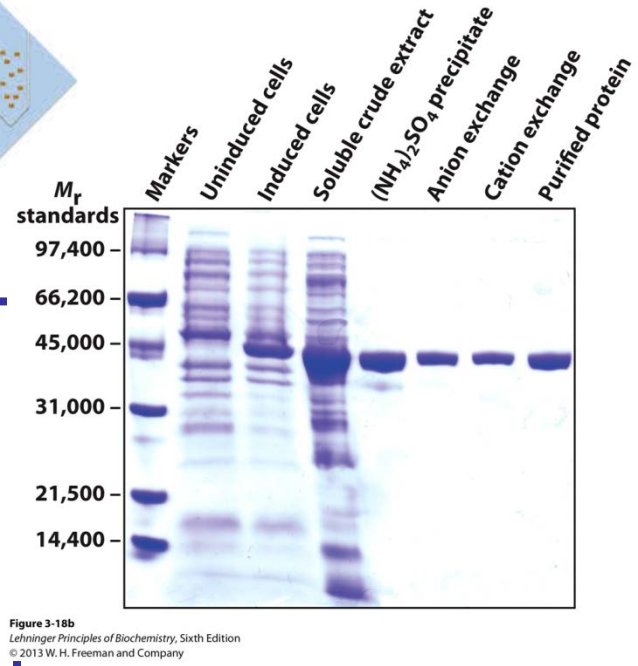


Figure 3-18b
Lehninger Principles of Biochemistry, Sixth Edition

- **all other quantities derived by calculation:**

sample vol × protein concn = **total protein**
(mL) (mg/mL) **(mg)**

sample vol × activity = **total activity**
(mL) (units/mL) **(units)**

$$\frac{\text{total activity (units)}}{\text{total protein (mg)}} = \textbf{specific activity (units/mg)}$$

- the higher the yield, the greater the recovery, the fewer times you'll need to get in the cold room and purify the enzyme

- the higher the purification factor, the purer the enzyme
  - the purification factor still doesn't give information about **how pure** it really is!

TABLE 3–5 **A Purification Table for a Hypothetical Enzyme**

Procedure or step	Fraction volume (mL)	Total protein (mg)	Activity (units)	Specific activity (units/mg)
1. Crude cellular extract	1,400	10,000	100,000	10
2. Precipitation with ammonium sulfate	280	3,000	96,000	32
3. Ion-exchange chromatography	90	400	80,000	200
4. Size-exclusion chromatography	80	100	60,000	600
5. Affinity chromatography	6	3	45,000	15,000

Note: All data represent the status of the sample *after* the designated procedure has been carried out. Activity and specific activity are defined on page 95.

Table 3-5
Lehninger Principles of Biochemistry, Sixth Edition

What properties of any enzyme should be determined?

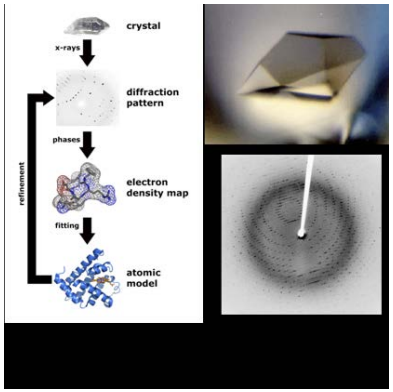
(i) molecular weight
- -electrospray mass spectrometry
- -gel filtration
- -analytical ultracentrifugation

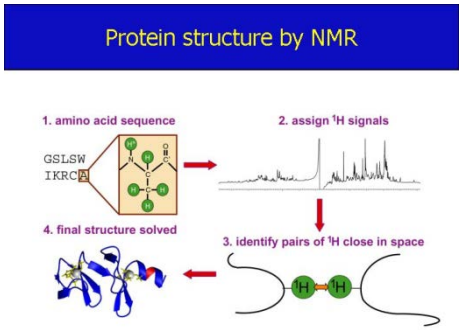
(ii) subunit structure
- -SDS-PAGE (± DTT)

(iii) -amino acid composition
- -6M HCl, 105°C, 24 h

(iv) -amino acid sequence
- -Edman degradation
- N-terminal sequencing
- -MS/MS mass spectrometry

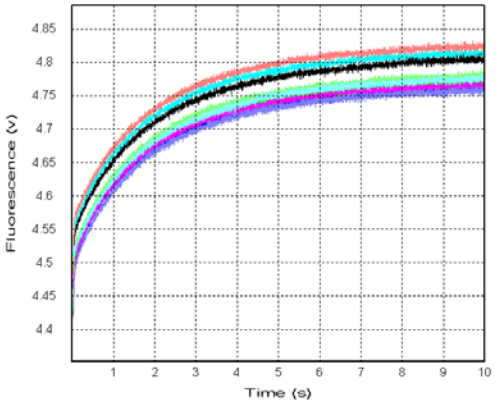
(v) -three dimensional structure
- -X-ray crystallography
- -NMR





6. Methods for Enzyme Activity Measurements

(a) Experimental approaches



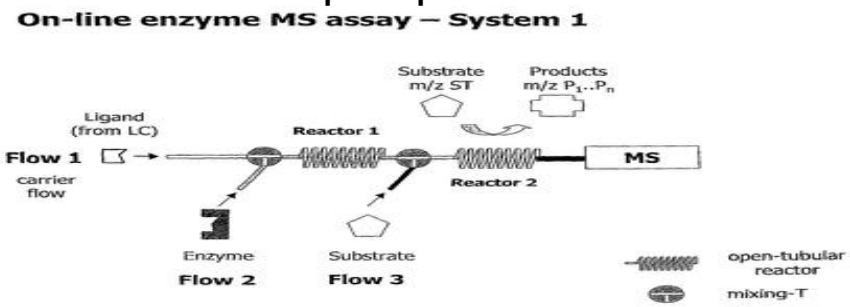
i. <u>spectrophotometric assays</u>- accurate and convenient
-fluorescence or absorbance changes

ii. <u>Release or uptake of H^+ (ΔpH)</u>- directly in buffered or weakly buffered solutions with a glass electrode
-restricted pH range (enzyme stability)
- indicator that changes its absorbance with protonation state
-**<u>pH stat technique</u>**-titrate the reaction mixture with either acid or base to keep the pH constant whilst recording the rate of addition
-ion-selective electrodes or gas electrodes

iii. <u>Automated assay procedures</u>

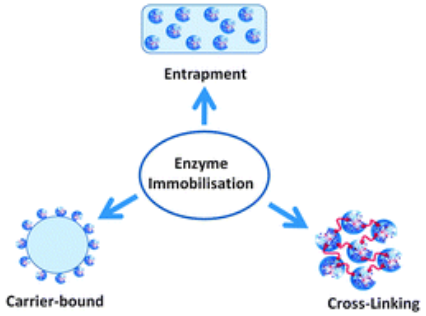
-automation of E assay can allow **large numbers of samples** to be processed rapidly and efficiently
-many involve the determination of P formation after a fixed time

(1) <u>continuous flow systems</u>-uses multi-channel pumps to mix reactants and determine P formation after a fixed time



(2) **immobilized enzymes**-enzymes are attached to a matrix (bead or surface)

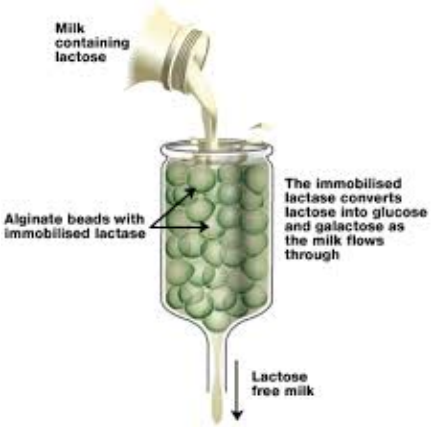
eg., trypsin-bead columns

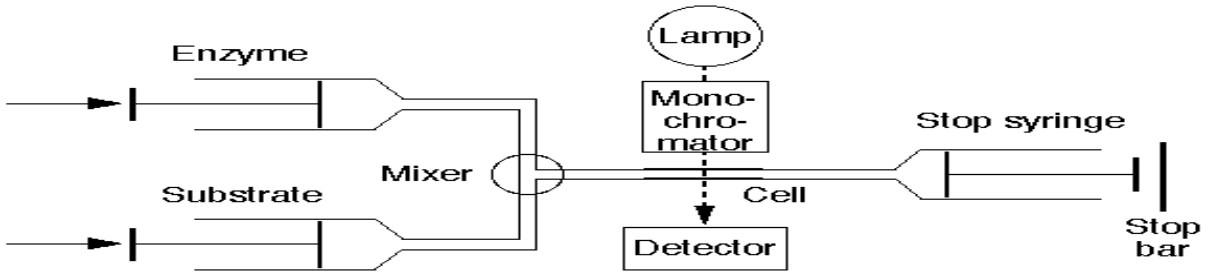


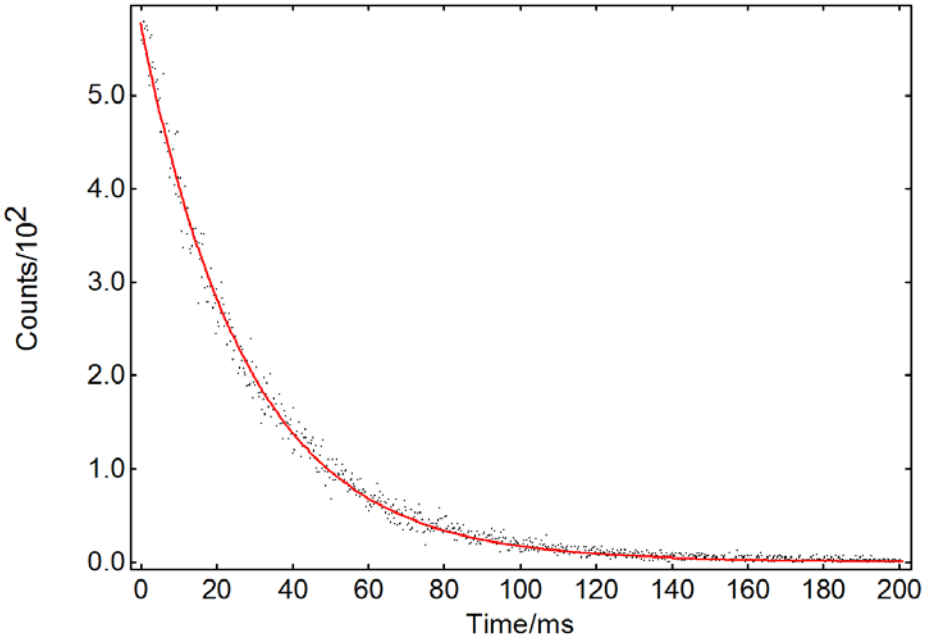
(3) **stopped-flow apparatus**

-enzyme mixed with substrate using a flow cell and detected with spectrometer

-allows rapid kinetic measurements (msec time scale)

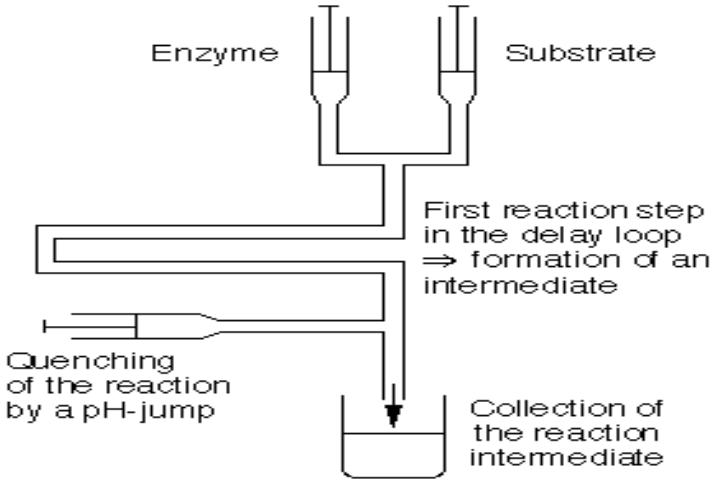






- Stopped-flow kinetic trace for the binding of a substrate to the enzyme
- Substrate binding causes quenching of the enzyme Trp fluorescence

4) **<u>quenched-flow apparatus</u>**-enzyme is mixed with substrate and quenched with acid or base in a second mixer



5) **multi-plate readers**-wavelength selection and temperature control coupled with rapid sample throughput

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