Chemistry 6720
Principles of Enzymology 1

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Principles of enzymology

Lecture 1: introduction to enzymology

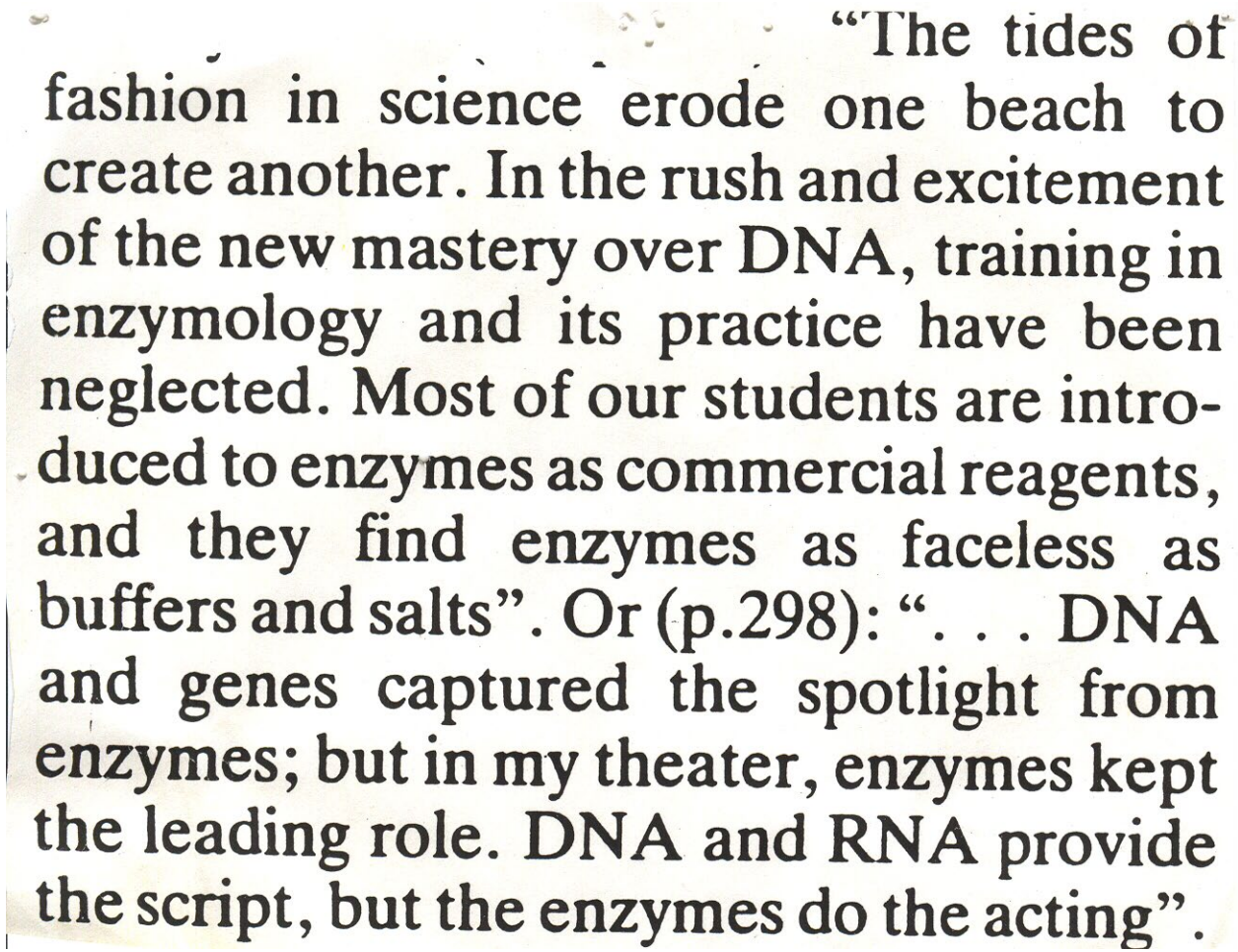
- What is enzymology and why is it important?
- Features of enzymes (overview)
- Enzyme classification
- Enzyme purification
- Enzymatic assays
- Chemical kinetics refresher
- Reaction mechanisms refresher

What is enzymology?

a branch of biochemistry that deals with enzymes, their nature, activity, and significance

Merriam Webster Dictionary

Why study enzymology?



"The tides of fashion in science erode one beach to create another. In the rush and excitement of the new mastery over DNA, training in enzymology and its practice have been neglected. Most of our students are introduced to enzymes as commercial reagents, and they find enzymes as faceless as buffers and salts". Or (p.298): ". . . DNA and genes captured the spotlight from enzymes; but in my theater, enzymes kept the leading role. DNA and RNA provide the script, but the enzymes do the acting".

Arthur Kornberg, Nobel prize, discovery of DNA polymerase

Features of enzymes

1. High reaction rates: 10^6 – 10^{12} x acceleration
2. Mild reaction conditions- water, low temp., low pressure, neutral pH
3. Reaction specificity
   - substrate (reactant), product
4. Capacity for regulation
   - substrates, products, metabolites, other proteins
   - allosteric control
   - covalent modification
   - enzyme synthesis

The rate of a chemical reaction depends on the relative free energies of:

- Initial reactants
- Stable intermediates
- Transition states

Catalysts can increase the rate of a chemical reaction by:

- Destabilizing initial reactants
- Stabilizing transition states
- Altering the mechanism of the reaction

General principles of enzyme catalysis

1. Proximity- Enzymes can accelerate a rate simply by holding the two reactants close together in appropriate orientation
2. General acid/base catalysis- make a potentially reactive group more reactive by increasing its intrinsic electrophilic or nucleophilic character
3. Electrostatic interactions- Enzymes stabilize the distribution of electrical charge in transitions state(s)
4. Enzymatic functional groups- covalent catalysis. Provide nucleophilic and electrophilic catalysts
5. Structural flexibility- induced fit
6. Weak interactions with substrate- “binding energy”

How to study an enzyme?

- Identify enzyme of interest
- Source material
- Purification of enzyme
- Develop and optimize enzymatic assay
- Kinetic characterization
- Mechanistic studies
- Amino acid sequence (gene)
- Structural characterization

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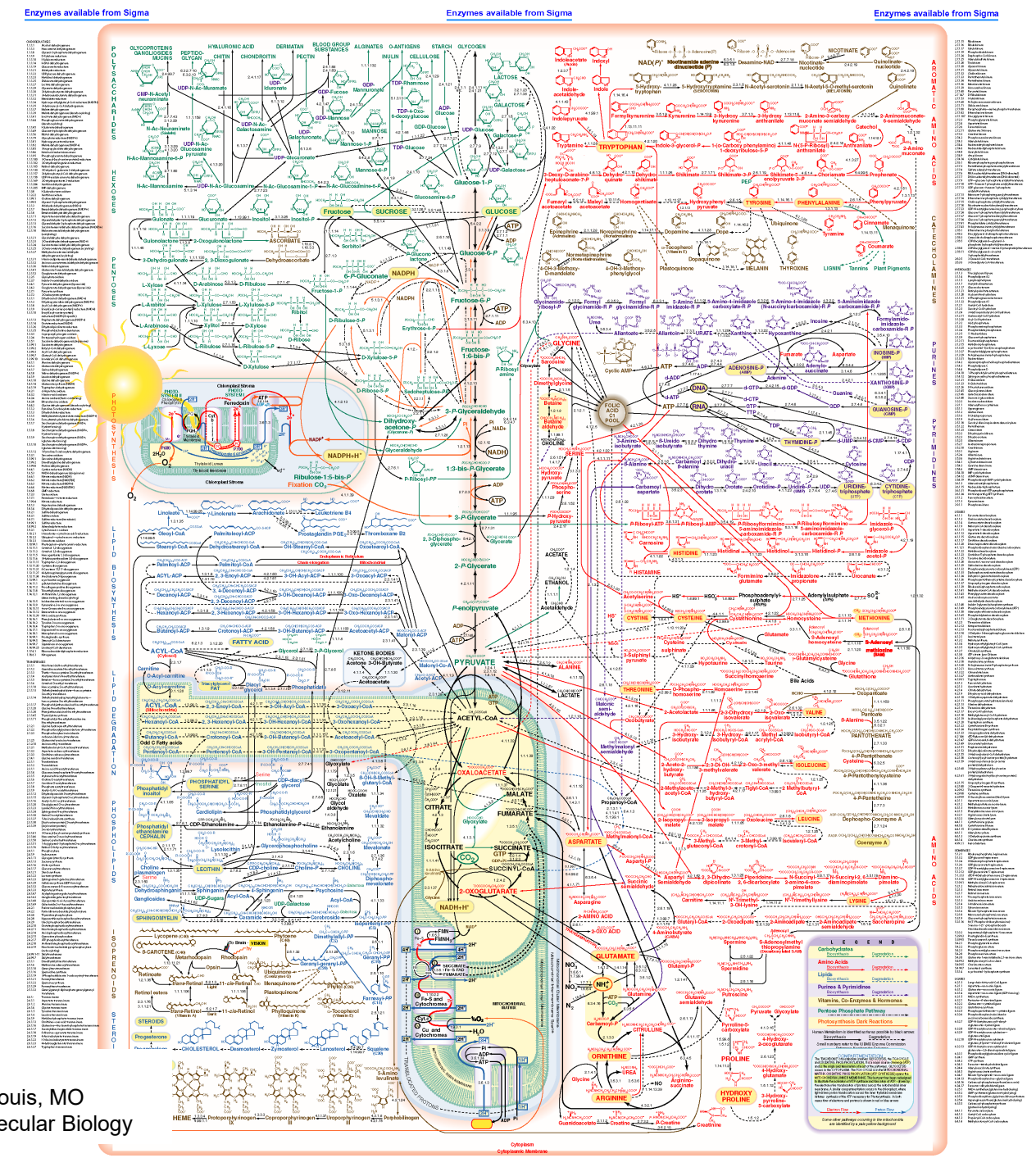

Designed by Donald Nicholson
Prepared and published by Sigma-Aldrich Co., St.Louis, MO

Four categories of enzymes (C. Walsh)

1. Group transfer reactions
2. Oxidations/reductions
3. Eliminations/isomerizations/rearrangements
4. Reactions that make or break C-C bonds

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International Enzyme Nomenclature

- Enzymes are assigned based on six classes of reactions that are a bit more confusing, yet allow more specific assignments

Enzyme classification

https://www.qmul.ac.uk/sbcs/iubmb/enzyme/

- Class I: oxidoreductases (EC 1.)
  - oxidation-reduction reactions
  - e^- donor : e^- acceptor oxidoreductase
- Class 2: Transferases (EC 2.)
  - phosphoryl, glycosyl, amino, etc
- Class 3: Hydrolases (EC 3.)
- Class 4: Lyases (EC 4.)
  - cleavage of C-C, C-O, C-N bonds, with elimination leaving an unsaturated residue. Includes additions to double bonds.
- Class 5: Isomerases (EC 5.)
  - racemases, epimerases, isomerases, tautomerases, mutases
- Class 6: Ligases (synthetases) (EC 6.)
  - joining of two molecules coupled to NTP hydrolysis

International Enzyme Nomenclature

Enzyme Nomenclature and Enzyme Commision Numbers

EC #	Class	# of sub-classes	Reactions catalyzed	Representative enzyme
1	oxidoreductases	22	Oxidation/reduction (e-transfer)	Alcohol dehydrogenase
2	transferases	9	Transfer of functional groups from one compound to another	hexokinase
3	hydrolases	13	Hydrolysis (group transfer to water)	chymotrypsin
4	lyases	7	Cleave C-C, C-O, C-N and other bonds by means other than (1) or (3)	Acetoacetate decarboxylase
5	isomerases	6	Changes within a single molecule; intramolecular rearrangement	Triose phosphate isomerase
6	ligases	6	Joining of two molecules forming new C-C, C-O, C-N, C-S bonds, using energy of NTP hydrolysis	Glutamine synthetase

International Enzyme Nomenclature

1. Group transfer

2. Oxidation/ reduction

3.Eliminations/ isomerizations

4. Reactions That make or Break C-C bonds

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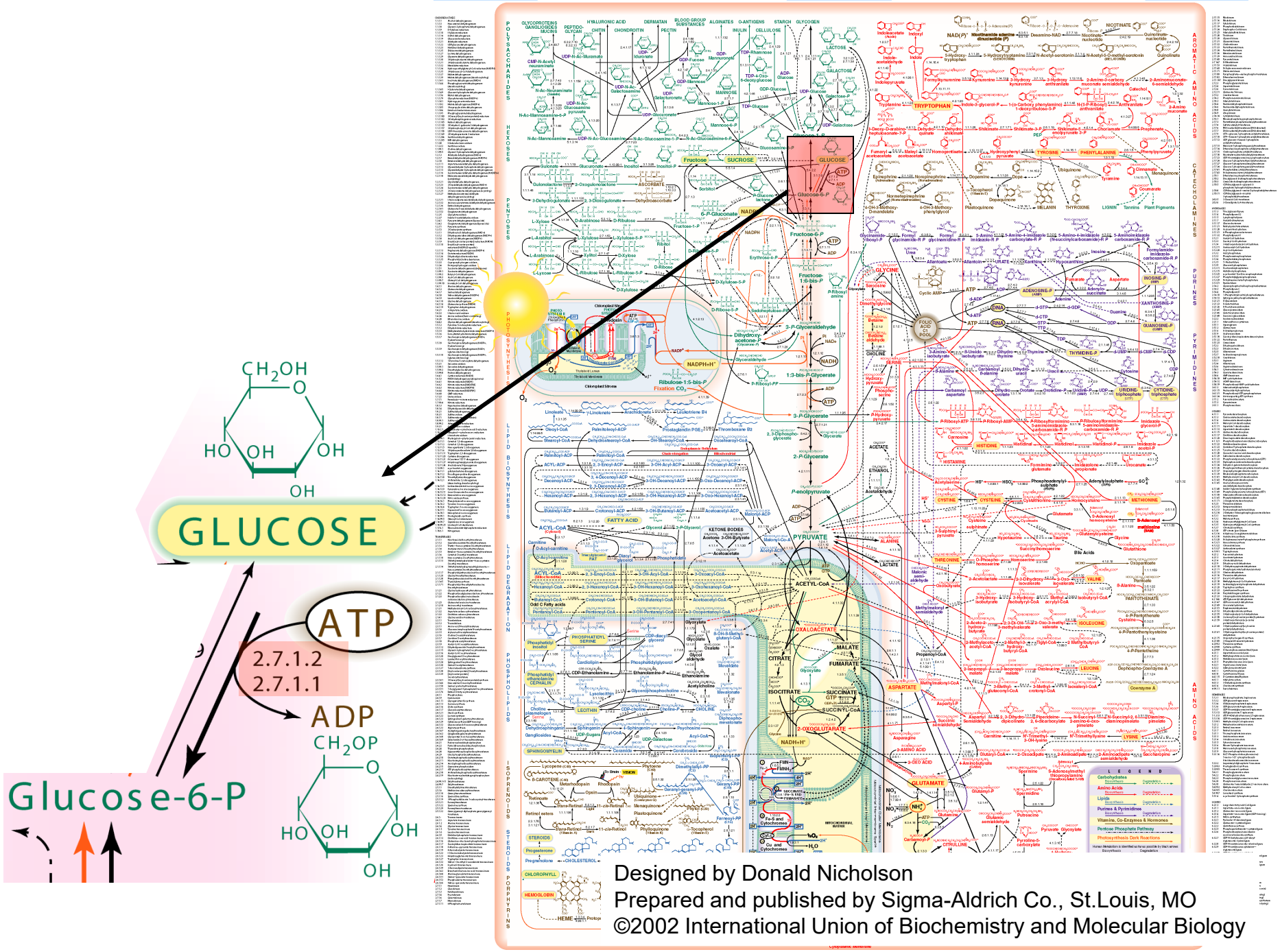
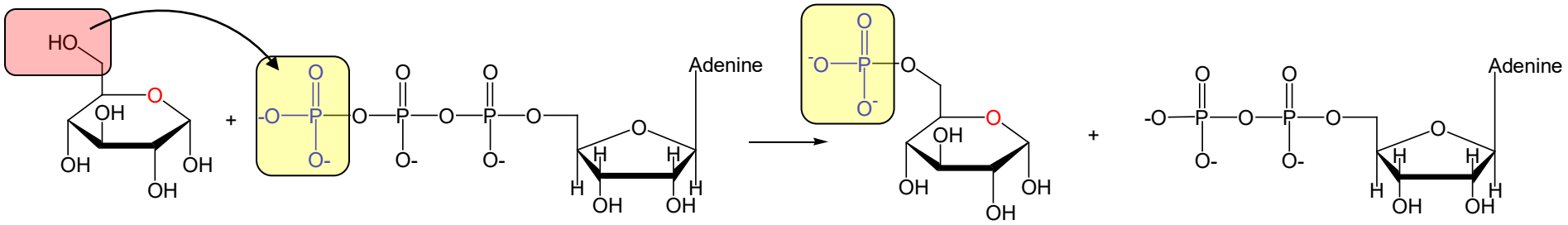
International Enzyme Nomenclature

- https://www.qmul.ac.uk/sbcs/iubmb/enzyme/
- Every enzyme can be assigned a unique four digit code based on six classes of enzymes
- e.g. “1.1.1.1” = alcohol dehydrogenase
- First digit: class of enzyme
- First three digits: classify reaction catalyzed
- Fourth digit: unique identifier for a particular enzyme
- Each enzyme is also assigned a systematic name describing the specific reaction catalyzed


Enzymes available from Sigma
Enzymes available from Sigma
Enzymes available from Sigma
GLUCOSE
ATP
2.7.1.2
2.7.1.1
ADP
Glucose-6-P
Designed by Donald Nicholson
Prepared and published by Sigma-Aldrich Co., St.Louis, MO
©2002 International Union of Biochemistry and Molecular Biology
Enzymes available from Sigma
Enzymes available from Sigma
Enzymes available from Sigma

International Enzyme Nomenclature: Hexokinase

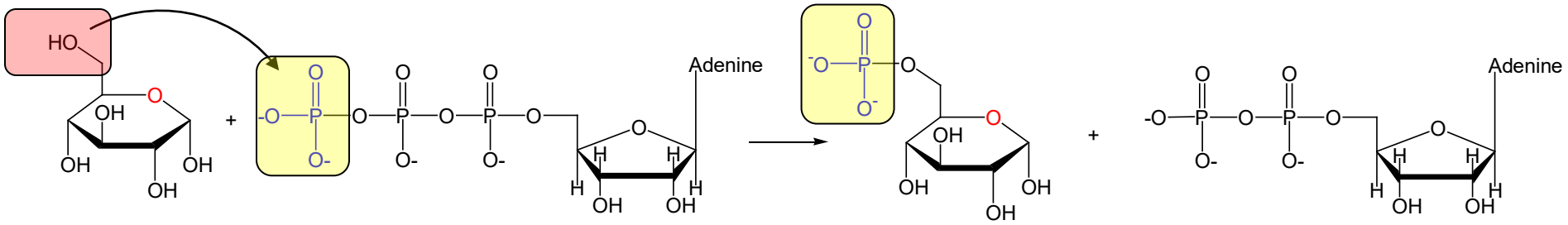
EC 2.7.1.1



- 2.X.X.X: Transfer of a functional group
- 2.7.X.X: Phosphorus-containing functional group is transferred
- 2.7.1.X: Phosphotransferases with an alcohol group as acceptor
- 2.7.1.1: “hexokinase” is the specific enzyme: glucose is acceptor, ATP is donor
- Systematic name: ATP:D-hexose 6-phosphotransferase

International Enzyme Nomenclature: Glucokinase

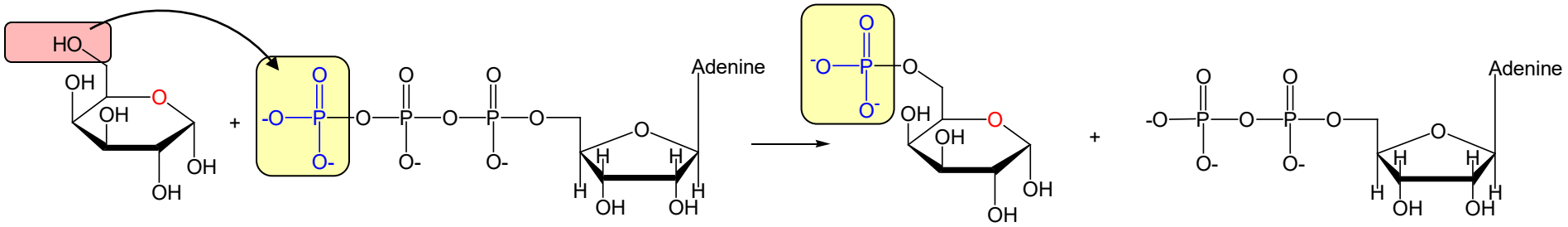
EC 2.7.1.2



- 2.X.X.X: Transfer of a functional group
- 2.7.X.X: Phosphorus-containing functional group is transferred
- 2.7.1.X: Phosphotransferases with an alcohol group as acceptor
- 2.7.1.2: “glucokinase” is the specific enzyme: glucose is acceptor, ATP is donor
- Systematic name: ATP:D-hexose 6-phosphotransferase
- Glucokinase is an “isozyme” of hexokinase

International Enzyme Nomenclature: Galactokinase

EC 2.7.1.6



- 2.X.X.X: Transfer of a functional group
- 2.7.X.X: Phosphorus-containing functional group is transferred
- 2.7.1.X: Phosphotransferases with an alcohol group as acceptor
- 2.7.1.6: “galactokinase” is the specific enzyme: galactose is acceptor, ATP is donor
- Systematic name: ATP:D-galactose 6-phosphotransferase

- Identify enzyme of interest
- Source material
- Purification of enzyme
- Develop and optimize enzymatic assay
- Kinetic characterization
- Mechanistic studies
- Amino acid sequence (gene)
- Structural characterization

Sources of enzymes

Purification of enzymes

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[1] Why Purify Enzymes?

By ARTHUR KORNBERG

'Don't waste clean thinking on dirty enzymes''

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- Source material
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Enzyme kinetics

- The branch of enzymology concerned with reaction rates, and factors that affect reaction rates
  - Magnitude of rate acceleration
  - Affinity and preference for substrates and products
  - Order of ligand binding and product release
  - Insights into chemical mechanism
  - Inhibitor profiles
    - Mechanistic insights
    - therapeutic value

A provocative quote:

"And for all the current and rather silly emphasis on structural biology, understanding enzymes means understanding catalysis and catalysis is concerned with kinetics, not structure: as Jeremy Knowles aptly remarked, ‘studying the photograph of a racehorse cannot tell you how fast it can run’ "

A. Cornish-Bowden, "Fundamentals of Enzyme Kinetics" (1995) Portland Press, London.

Enzyme kinetics

Before you can do enzyme kinetics, you need an optimized enzyme assay to measure activity

Enzymatic assays

- Units
- Assay method
- Linearity
- Optimization of assay conditions

Assay methods

- Spectrophotometry
- Fluorescence
- Selective electrodes
- Chromatography (LC, TLC, GC, GC-MS)
- Radioactivity
- Chemical derivatization
- Coupled assays (examples?)
- others

continuous vs. discontinuous assay

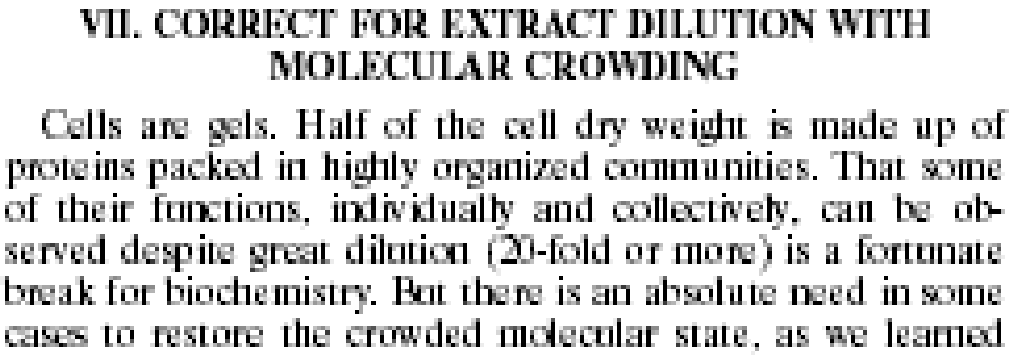
Linearity of enzyme assay

- Double [E], double the rate
- How long is [P] vs. *t* linear?

"linearity" is linked to "optimization" and "stabilization"

Optimization of enzyme assay

- pH
- Choice of buffer
- Temperature
- exogenous cofactor
- Stabilizing agents
  - reductant, metal ion chelator, bulking agent, salt, lipid, etc, etc.

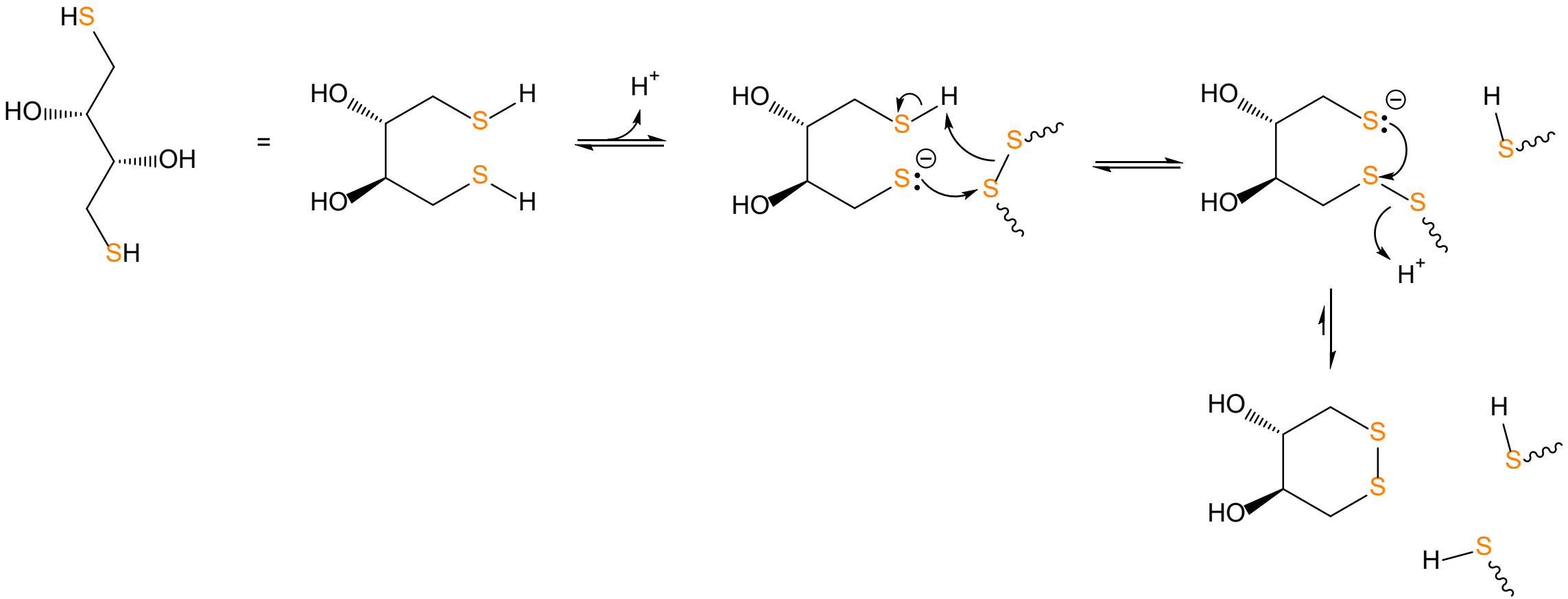


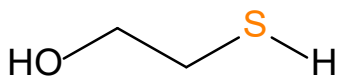
VII. CORRECT FOR EXTRACT DILUTION WITH MOLECULAR CROWDING

Cells are gels. Half of the cell dry weight is made up of proteins packed in highly organized communities. That some of their functions, individually and collectively, can be observed despite great dilution (20-fold or more) is a fortunate break for biochemistry. But there is an absolute need in some cases to restore the crowded molecular state, as we learned

- Lability to oxygen???
  - Cells are largely anaerobic and a reducing environment

Mechanism of disulfide bond reduction by DTT





Lecture 2

- Enzyme assays (cont):
  - Calculating specific activity from spectrophotometric assay data
  - Continuous vs. discontinuous assays
  - Coupled spectrophotometric assays
- Chemical kinetics refresher
- Begin steady state kinetics section

Assay methods

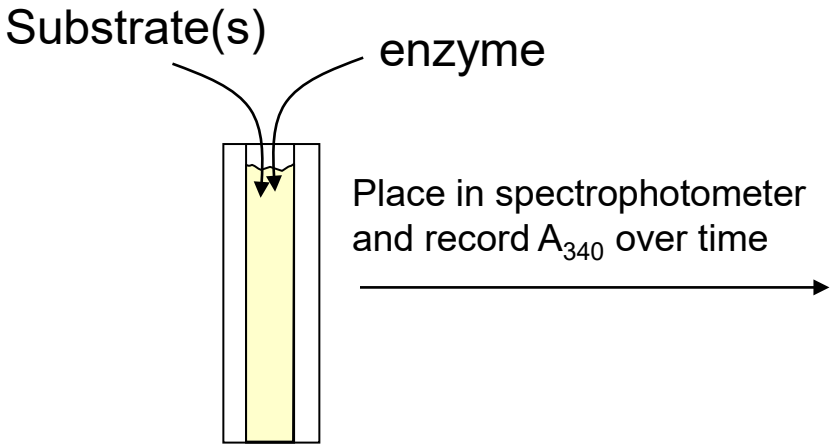
- Spectrophotometry
- Fluorescence
- Selective electrodes
- Chromatography (LC, TLC, GC, GC-MS)
- Radioactivity
- Chemical derivatization
- Coupled assays (examples?)
- others

continuous vs. discontinuous assay

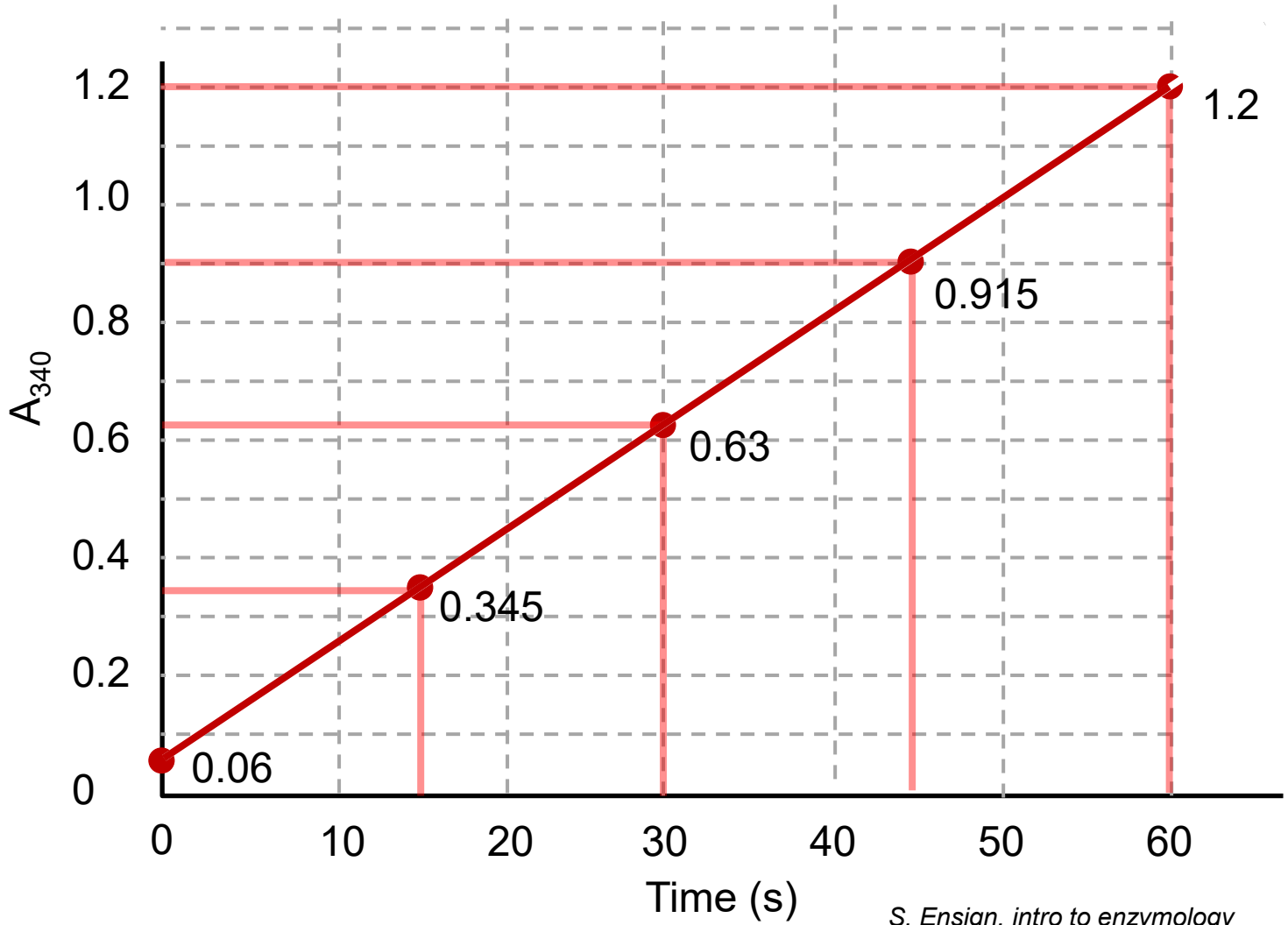
How to determine specific activity from spectrophotometric data

- Determine $\Delta A/\Delta T$ from progress curves
  - Be sure assays are linear over assay time course
  - Be sure doubling enzyme doubles slope of progress curves
- Convert $\Delta A/\Delta T$ to units of μmol/min using Beer's law ($A = \varepsilon cl$) and dimensional analysis
- Example: Alcohol dehydrogenase-

You have isolated an alcohol dehydrogenase that catalyzes the oxidation of ethanol to acetaldehyde. You carry out spectrophotometric assays in a cuvette to which you have added 0.75 mL of buffer, 0.1 mL of 100 mM ethanol and 0.1 mL of NAD+. To initiate the reaction you add 0.050 mL of a 3.08 mg/mL solution of your purified enzyme. The resulting progress curve for increase of A340 over time remains linear for at least one minute. When you repeat the assay in the same total volume with twice the amount of enzyme, the slope of the progress curve doubles. What is the specific activity of your enzyme in units of μmol/min/mg protein?



$$\frac{\Delta A}{\Delta t} = \frac{A_{t_2} - A_{t_1}}{t_2 - t_2}$$



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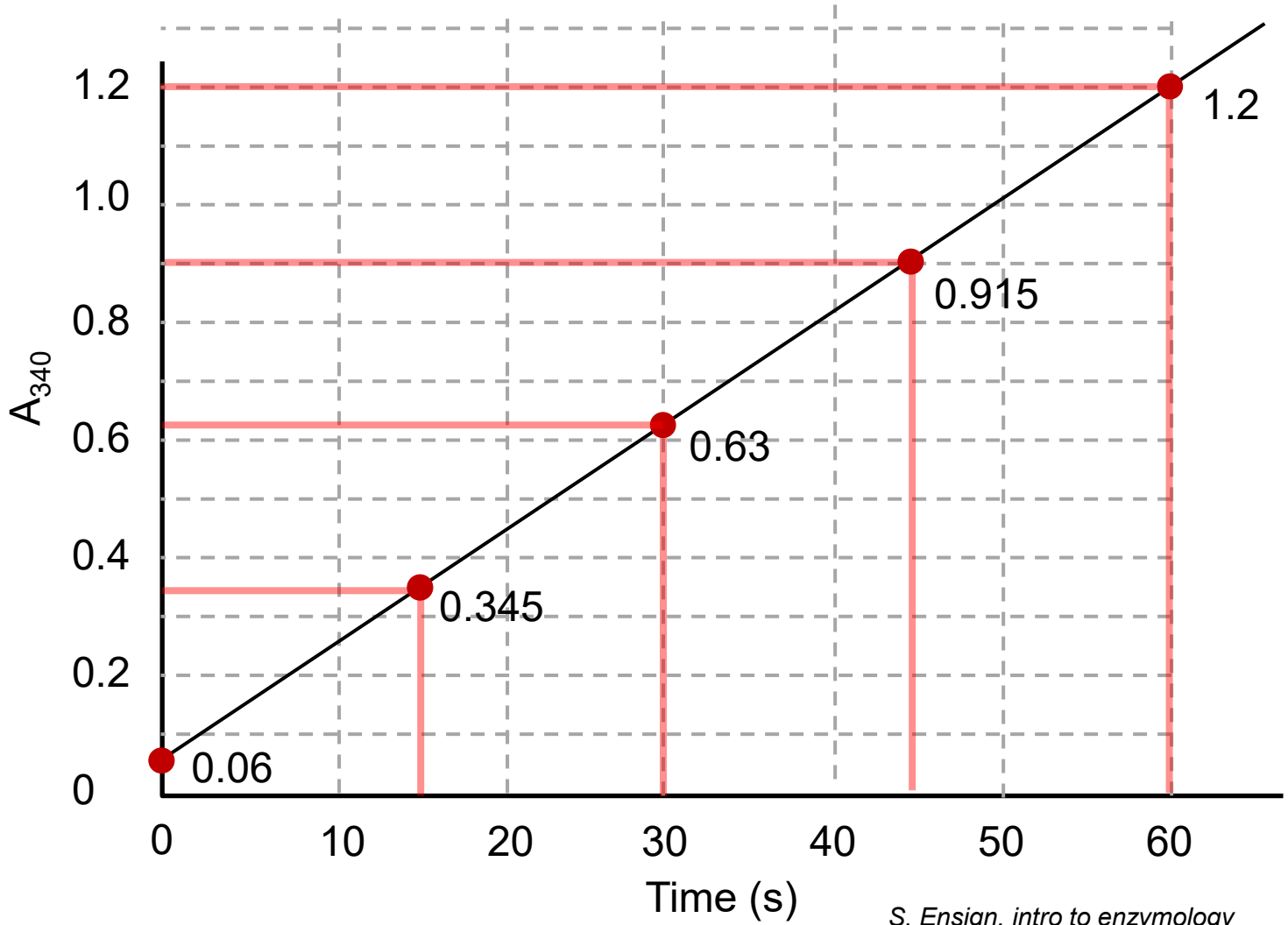

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$\Delta A/\Delta T = 0.0190/s$

continuous vs. discontinuous assay

Rubisco activity: how measure?

ATPase activity- how measure?

Glutamine synthetase activity: How measure?

Coupled enzyme assays

- Useful for enzymes where no direct continuous assay is available, but where a product can be linked to a reaction involving NAD^+/NADH
- Common coupling enzymes: pyruvate kinase, lactate dehydrogenase

Chemical kinetics refresher

- “Overall reaction” vs. “elementary steps” in the overall reaction
- Intermediates appear in elementary steps
- The rate law for the overall reaction must be experimentally determined
- Rate laws for elementary steps can be deduced from the stoichiometry of reactants/intermediates in the elementary step

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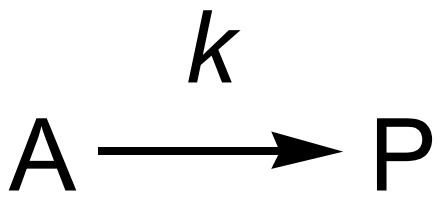
Chemical kinetics refresher



$$v = -\frac{d[\mathrm{A}]}{dt} = \frac{d[\mathrm{P}]}{dt} = k\ [\mathrm{A}]^{\mathrm{n}}$$

A first order reaction

$$A \xrightarrow{k} P \qquad v = -\frac{d[A]}{dt} = \frac{d[P]}{dt} = k\,[A]^1$$

Units of concentration are M

Units of rate are $M \cdot s^{-1}$

Units of *k* are s^{-1}

$$\ln[A] = \ln[A]_o - kt \qquad [A] = [A]_o e^{-kt}$$

$$t_{1/2} = \frac{\ln 2}{k} = \frac{0.693}{k}$$

A second order reaction

$$2A \xrightarrow{k} P \qquad v = -\frac{d[A]}{dt} = \frac{d[P]}{dt} = k\,[A]^2$$

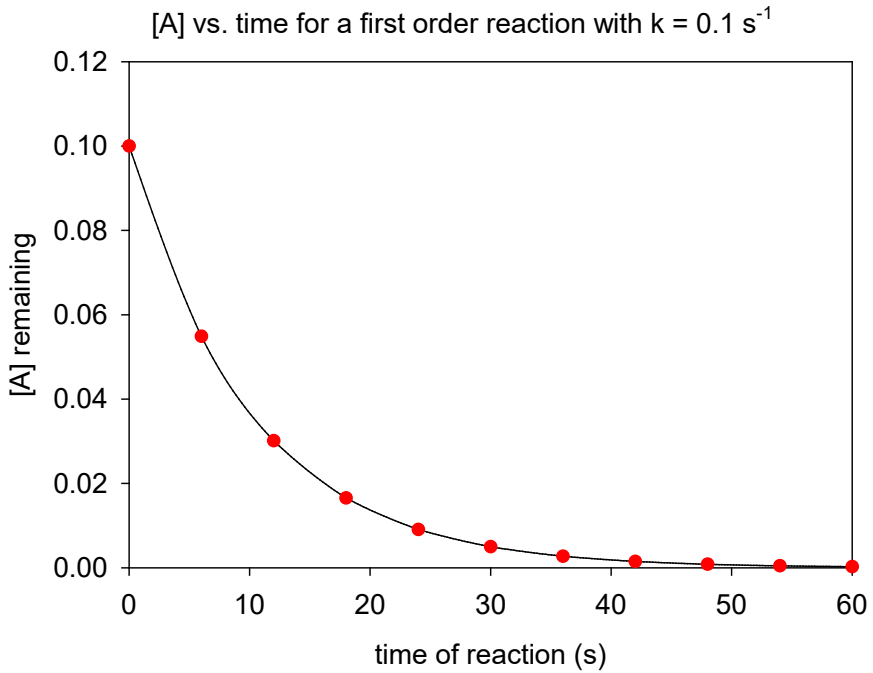
Units of concentration are M

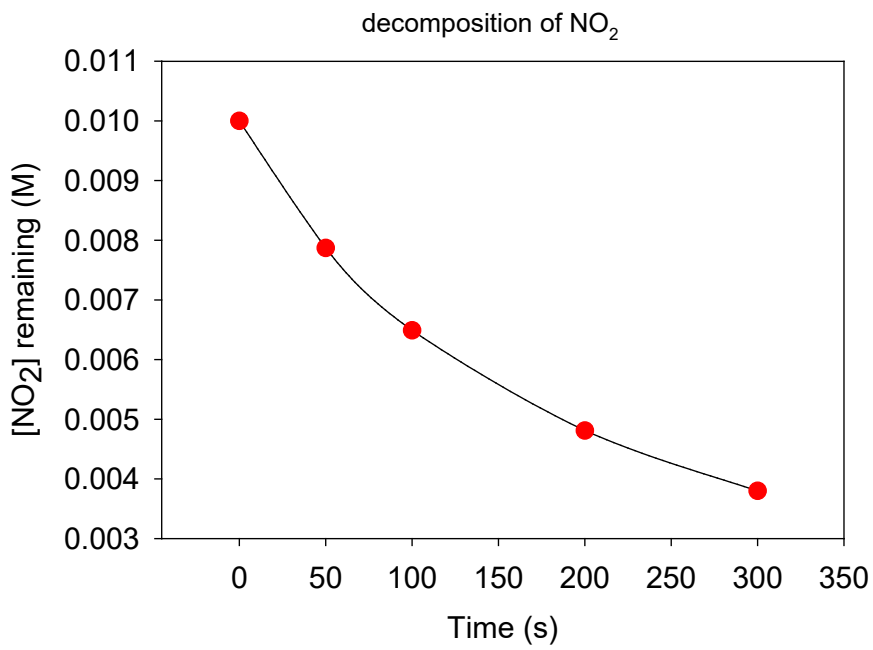
Units of rate are $M \cdot s^{-1}$

Units of k are $M^{-1} \cdot s^{-1}$

$$\frac{1}{[A]} = \frac{1}{[A]_0} + kt$$

[A] vs. t for 1st and 2nd order reactions





$$\ln[\mathrm{A}] = \ln[\mathrm{A}]_o - kt$$

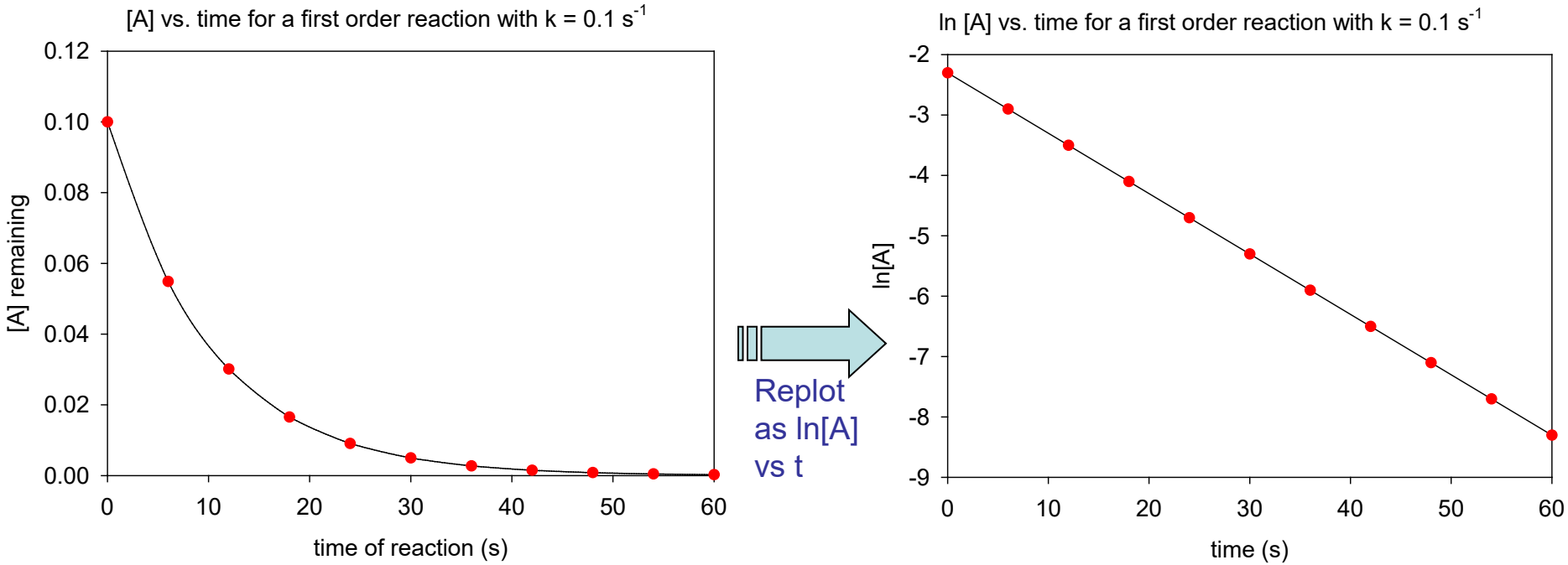
$$\frac{1}{[A]} = \frac{1}{[A]_0} + kt$$

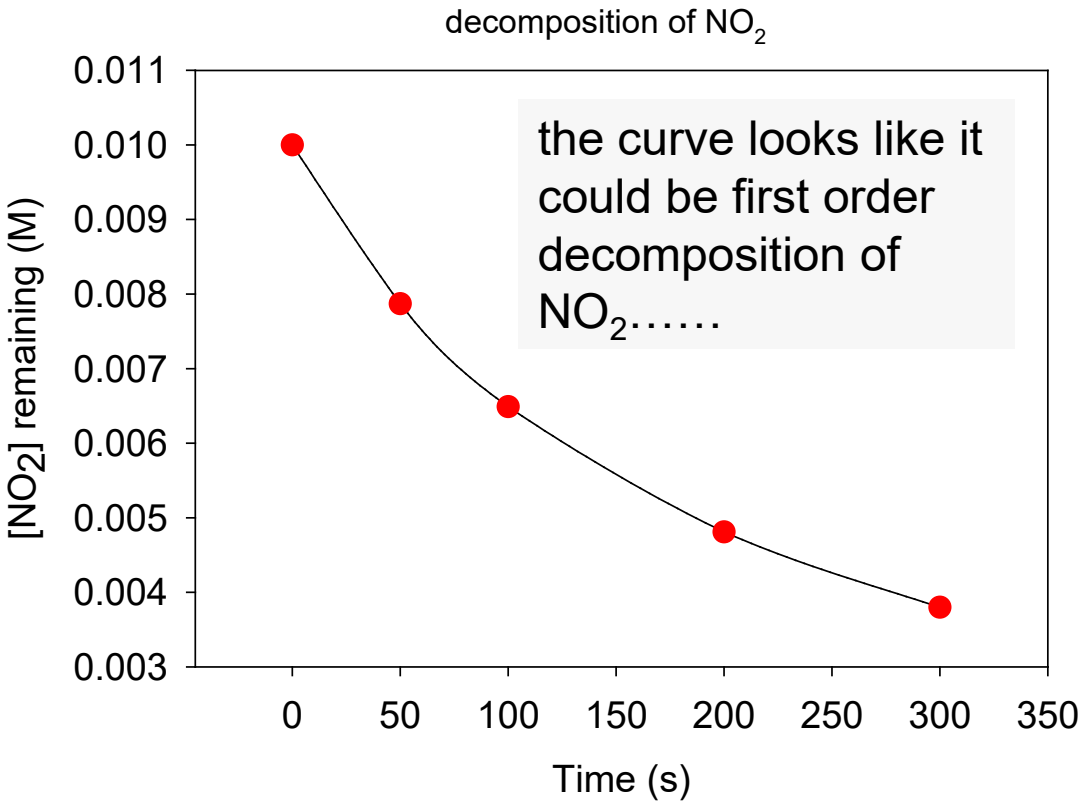
- A plot of ln[A] vs. time will be linear if the reaction is first order
- A plot of 1/[A] vs. t will be linear if the reaction is second order

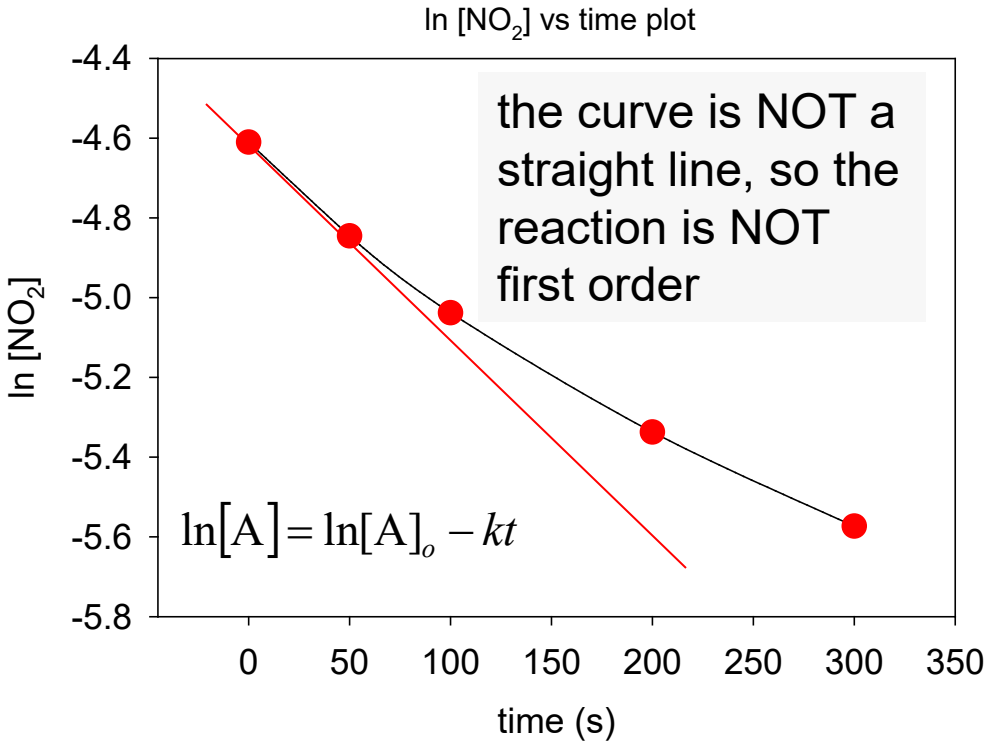
$$[A] = [A]_0 \, e^{-kt}$$

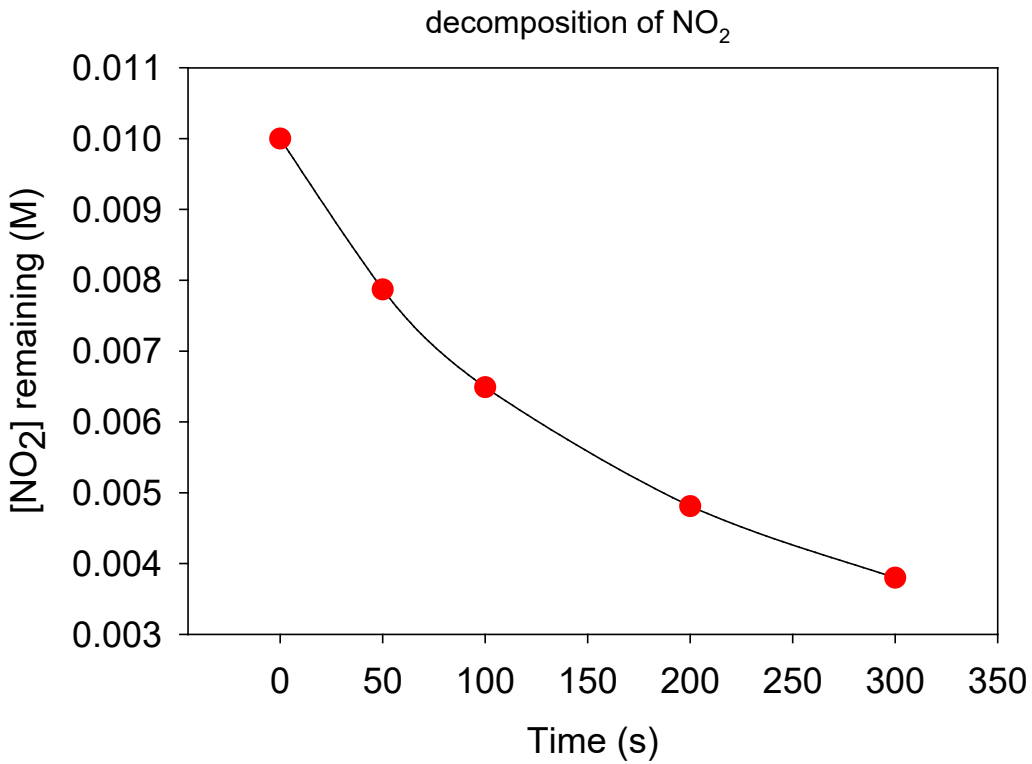
$$\ln[A]_t = -kt + \ln[A]_0$$

$$y = mx + b$$




decomposition of NO2
[NO2] remaining (M)
0.011
0.010
0.009
0.008
0.007
0.006
0.005
0.004
0.003
0
50
100
150
200
250
300
350
Time (s)
the curve looks like it could be first order decomposition of NO2……


ln [NO2] vs time plot
ln [NO2]
-4.4
-4.6
-4.8
-5.0
-5.2
-5.4
-5.6
-5.8
0
50
100
150
200
250
300
350
time (s)
the curve is NOT a straight line, so the reaction is NOT first order
ln[A] = ln[A]o − kt


decomposition of NO_2
[NO2] remaining (M)
0.011
0.010
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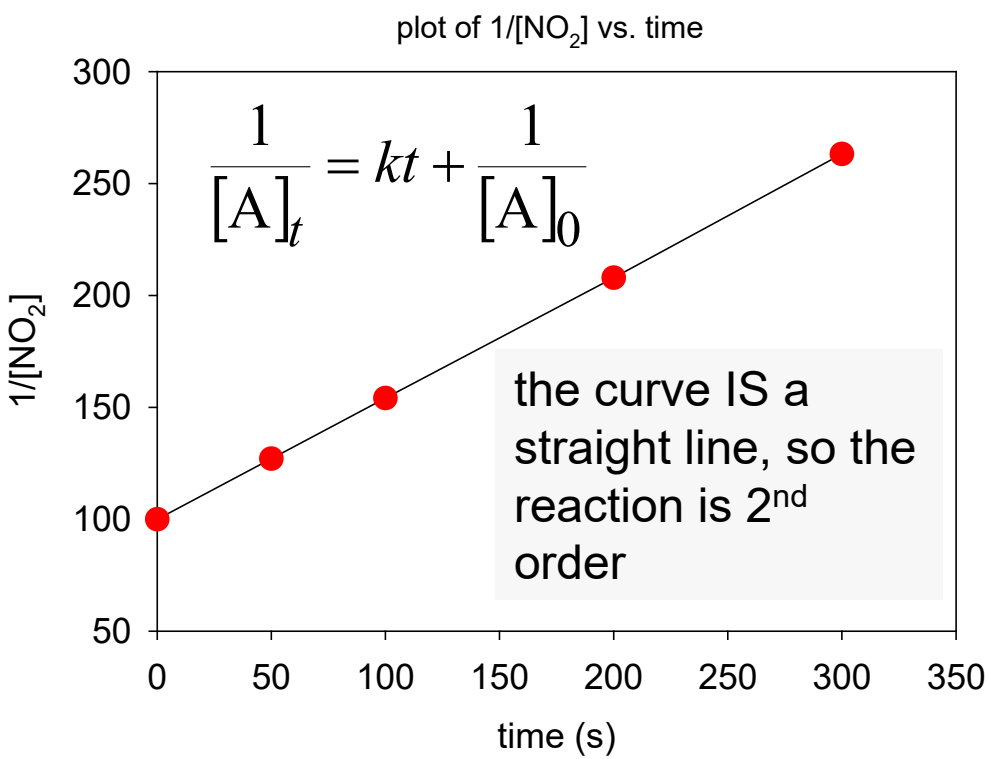
0.003
0
50
100
150
200
250
300
350
Time (s)


plot of 1/[NO_2] vs. time
$\frac{1}{[A]_t} = kt + \frac{1}{[A]_0}$
1/[NO2]
300
250
200
150
100
50
0
50
100
150
200
250
300
350
time (s)
the curve IS a straight line, so the reaction is 2nd order

- Identify enzyme of interest
- Source material
- Purification of enzyme
- Develop and optimize enzymatic assay
- Kinetic characterization
- Mechanistic studies
- Amino acid sequence (gene)
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Reaction mechanism

https://en.wikipedia.org/wiki/Reaction_mechanism

"A reaction mechanism is the step by step sequence of elementary reactions by which overall chemical change occurs. A chemical mechanism is a theoretical conjecture that tries to describe in detail what takes place at each stage of an overall chemical reaction. The detailed steps of a reaction are not observable in most cases. The conjectured mechanism is chosen because it is thermodynamically feasible, and has experimental support in isolated intermediates (see next section) or other quantitative and qualitative characteristics of the reaction. It also describes each reactive intermediate, activated complex, and transition state, and which bonds are broken (and in what order), and which bonds are formed (and in what order). A complete mechanism must also explain the reason for the reactants and catalyst used, the stereochemistry observed in reactants and products, all products formed and the amount of each."

In order to understand and draw reaction mechanisms, you must be able to push electrons!

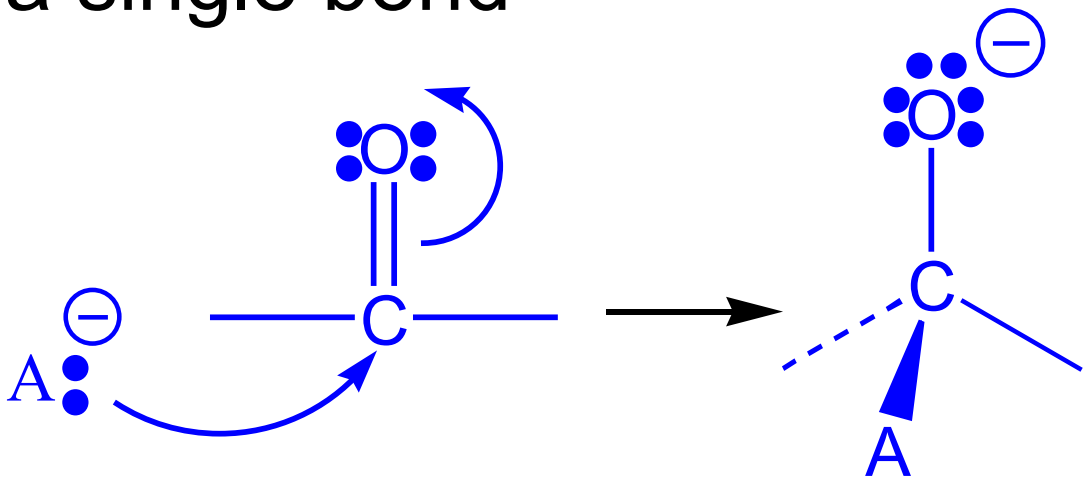
- Electron pushing: using arrows to show movement of electrons
- Arrows used to show redistribution of e^- density are drawn from a position of high e^- density to an e^- deficient position
  - From neg. charge/lone pairs towards (+) charge or (+) end of dipole
  - From nucleophiles to electrophiles

In order to understand and draw reaction mechanisms, you must be able to push electrons!

- A double headed arrow = an e^- pair

- A single headed arrow = a single e^-

- A lone e^- pair ⟷ a single bond



S_N2 reaction mechanism