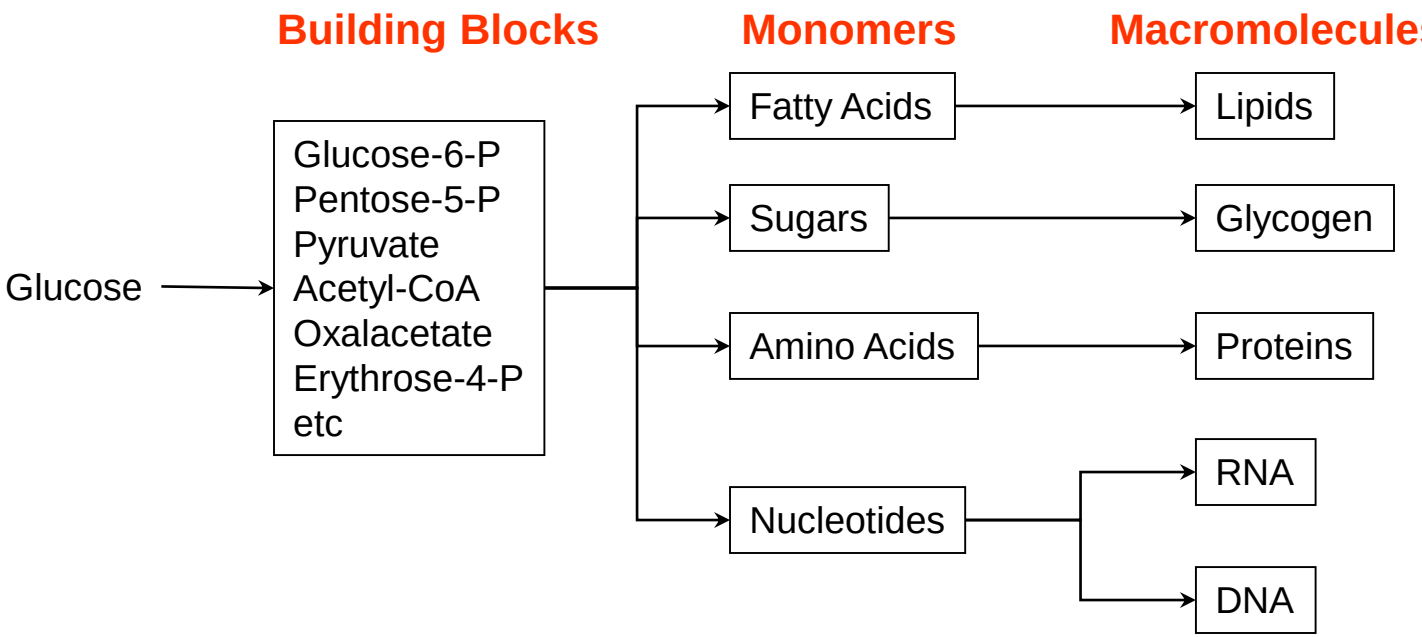
Extracellular Enzymes Lab

Biochemistry

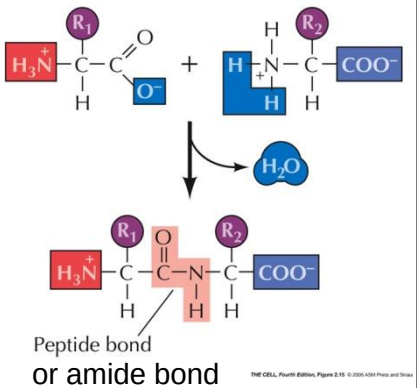
- All organisms convert small organic compounds, such as glucose, into monomers required for the production of macromolecules; e.g.,



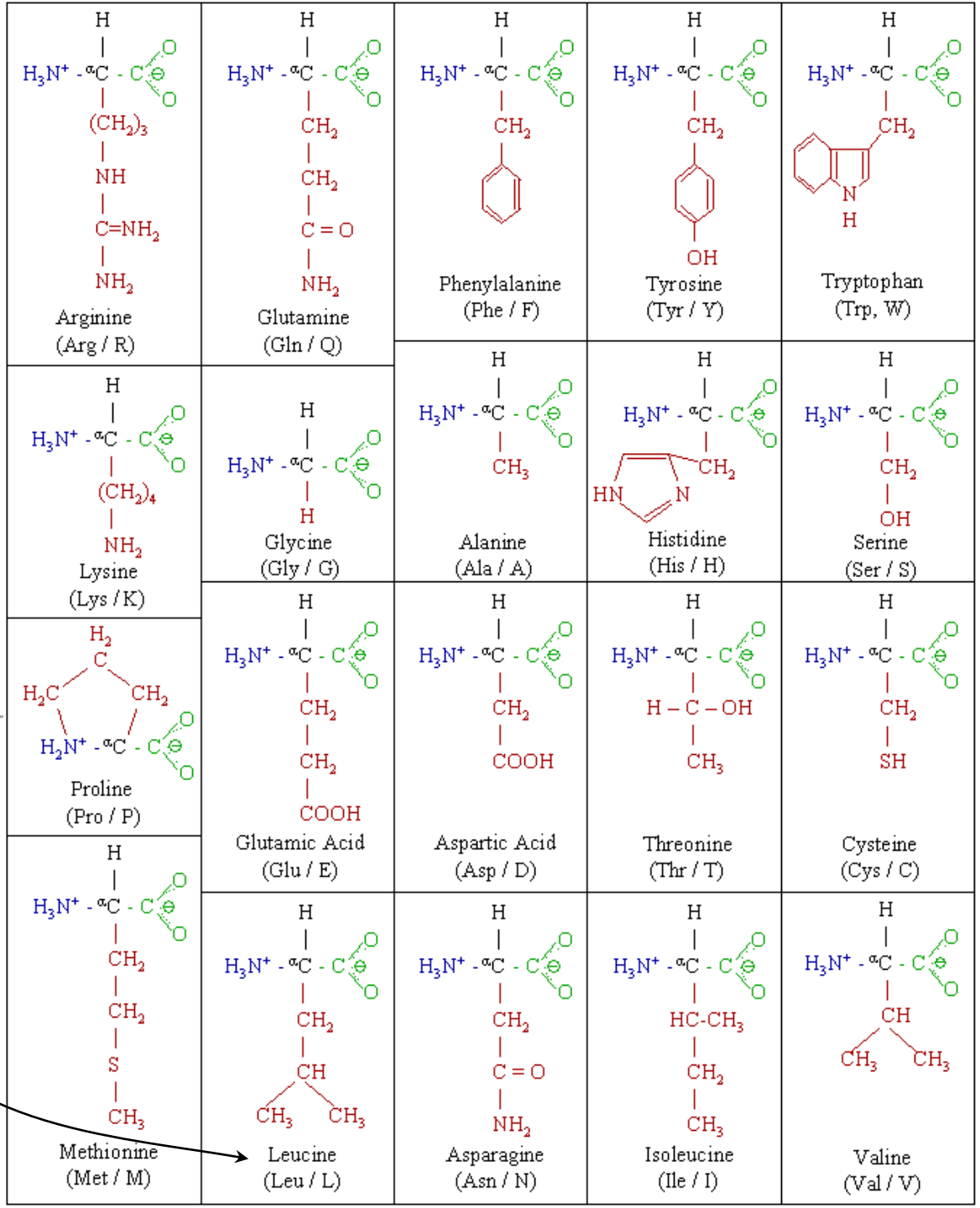
Assigned readings (in addition to Brock):

- Lehninger (1979) Biochemistry Ch 8. Enzymes: kinetics and inhibition
- H.-G. Hoppe (1993) Aquatic microbial ecology Ch 48 Use of fluorogenic model substrates for extracellular activity measurements of bacteria

Amino Acids

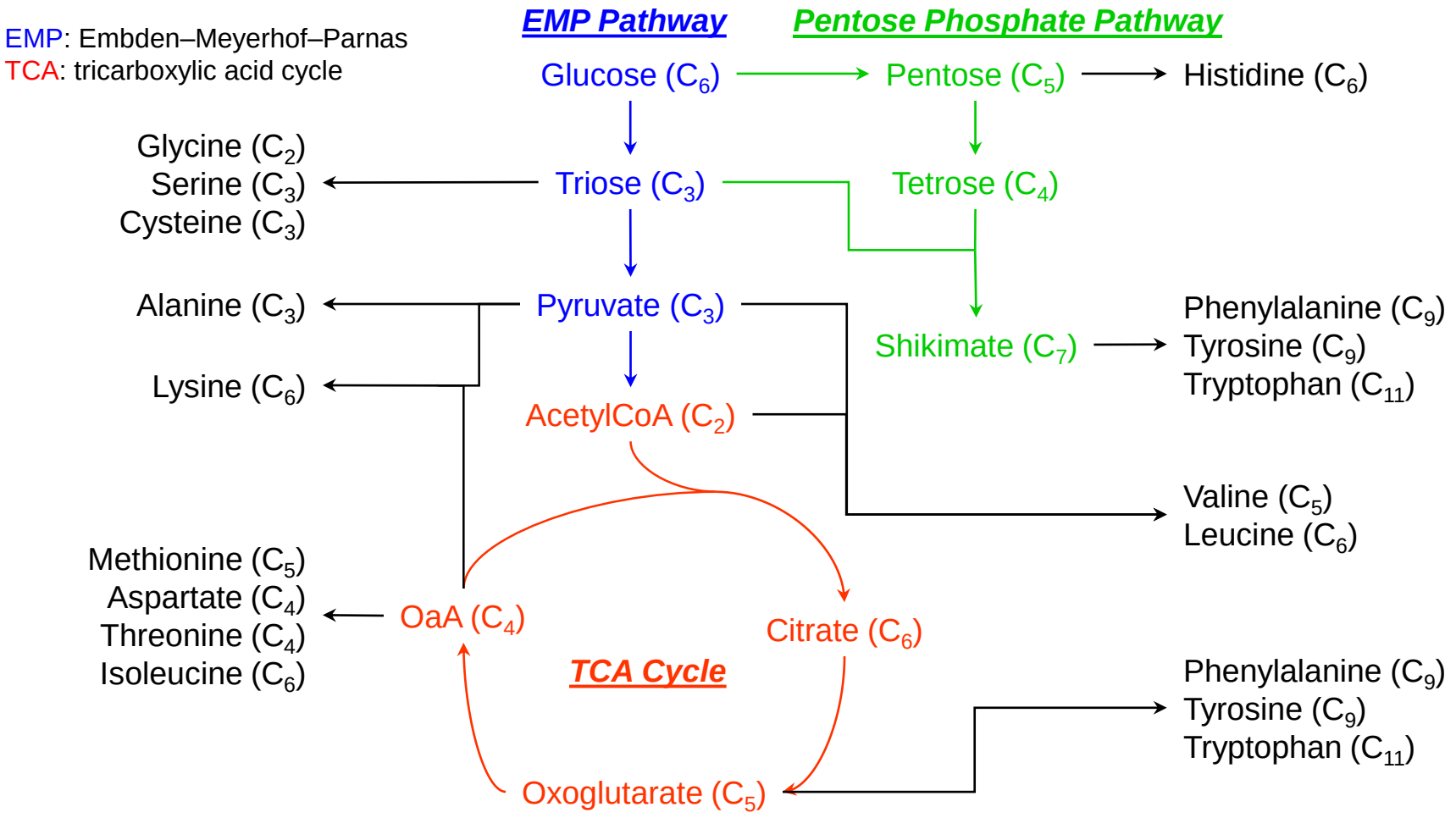


Bacterial Production Lab used Leucine



Metabolism

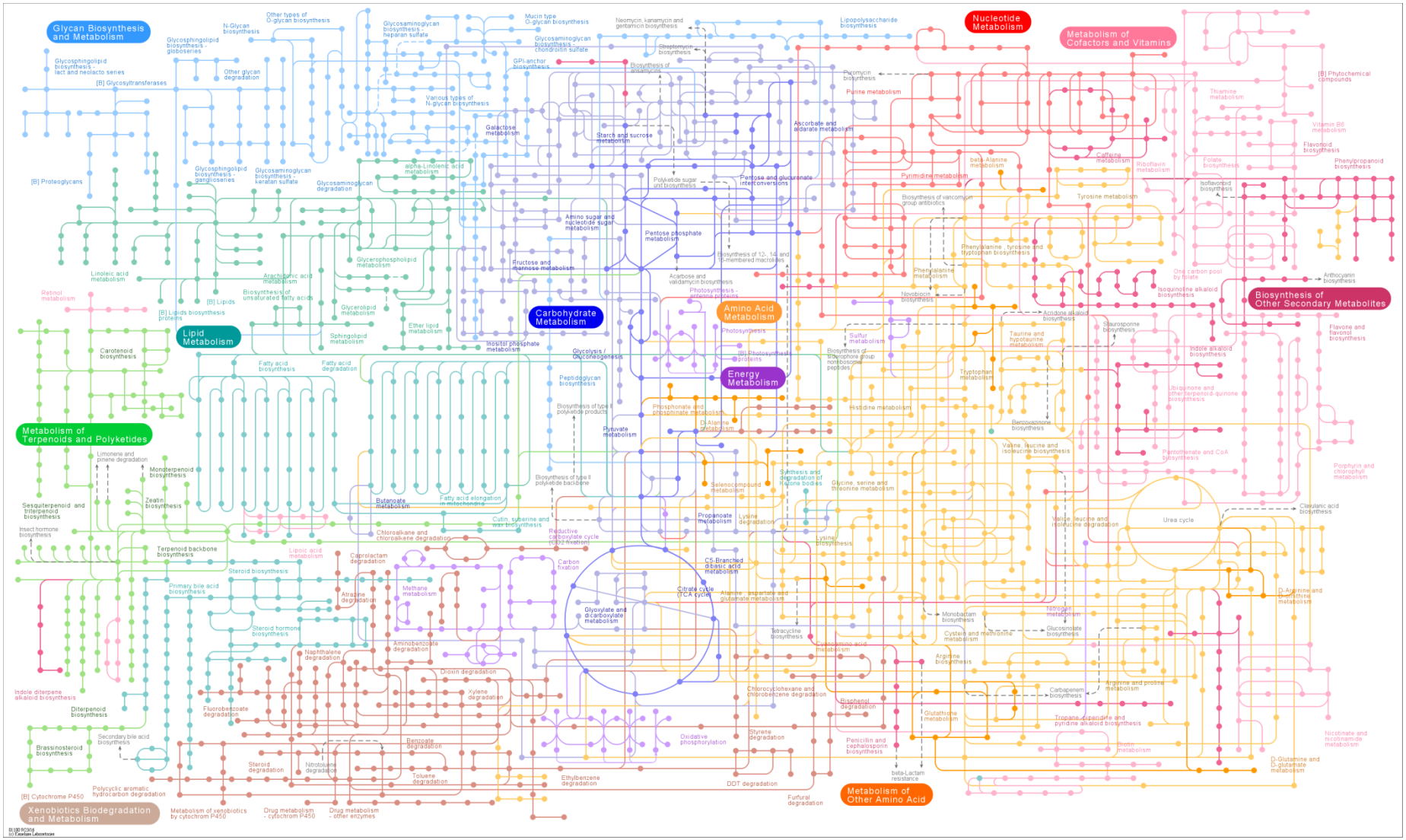
The synthesis of building block compounds, monomers and macromolecules from glucose (and other simple compounds, such as CO_2) is conducted by the metabolic reactions of the cell, such as the highly abbreviated synthesis of amino acids shown here:



All of these reactions, of which there are more than 1000, are catalyzed by enzymes.

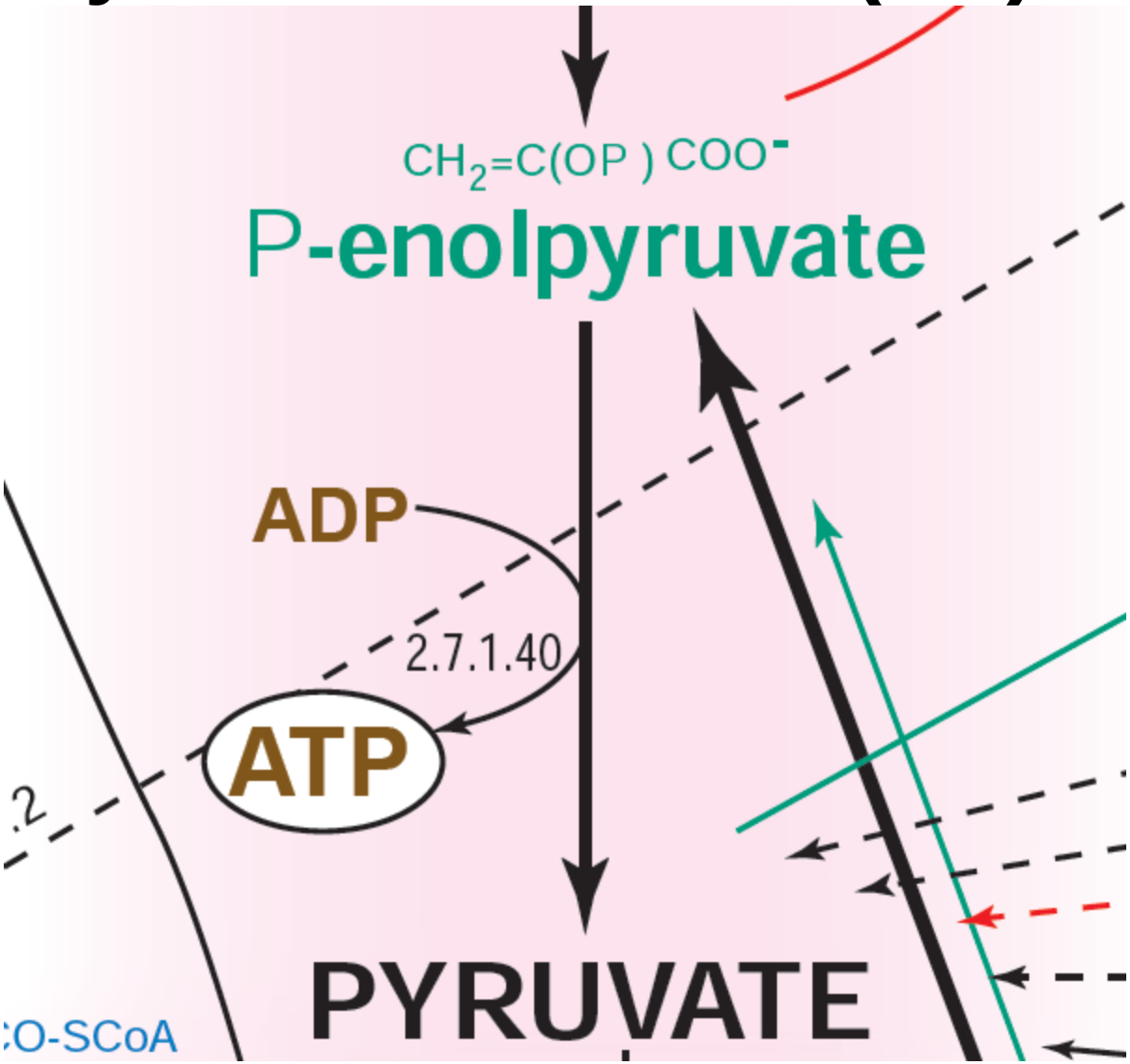
More Complete Metabolic Network

From KEGG (Kyoto Encyclopedia of Genes and Genomes) http://www.genome.jp/kegg/



22nd Edition Designed by Donald E. Nicholson, D.Sc., The University of Leeds, England – and Sigma-Aldrich
Product No. M 3907

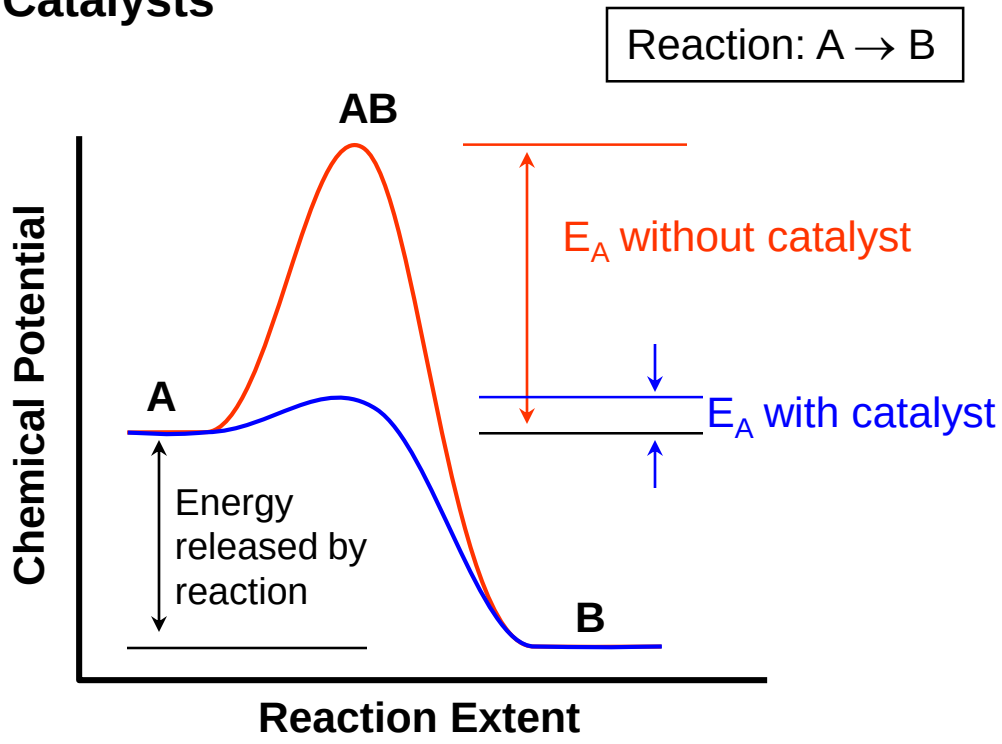
The Enzyme Commission (EC) number



Enzymes

- Enzymes are large proteins that all organisms synthesize to catalyze metabolic reactions.
- Enzymes are typically highly specific, converting only one substrate to one product.
- Almost all reactions that occur within the cell, including energy production (catabolism) and biosynthesis (anabolism), are catalyzed by enzymes.
- Reactions that are thermodynamically unfavorable (i.e., endoergic) require an energy source, such as ATP to proceed.

Catalysts

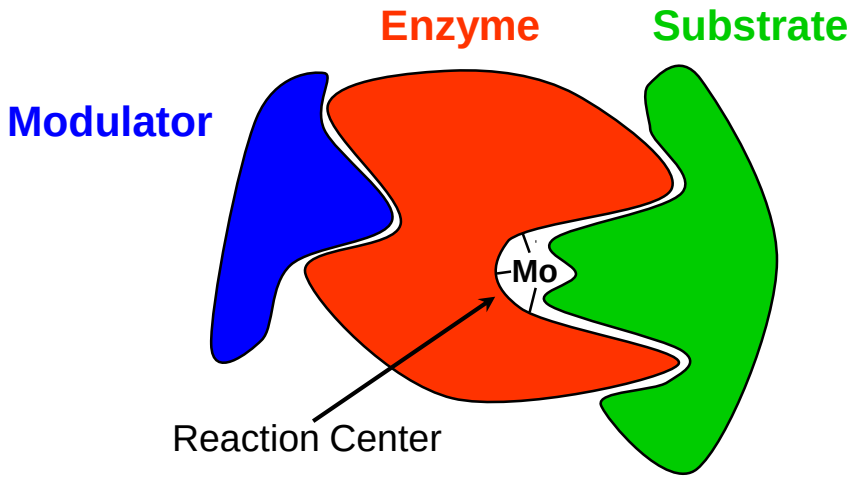


- Activation energy, E_A, must be supplied to most reactions in order for them to proceed.
- A catalyst lowers the activation energy, allowing a reaction to proceed at lower temperatures.
- Catalysts are neither consumed nor produced in the reaction.
- Enzymes are a class of catalysts

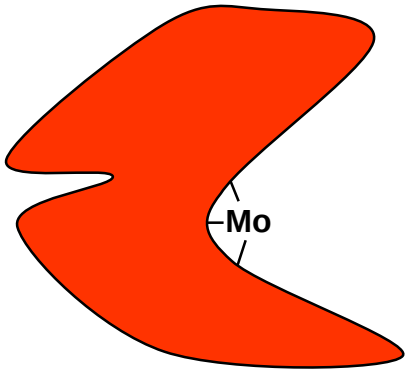
Brock: pp. 116-117, Sec. 5.5

Enzyme Catalysis

- The sequence of amino acids that comprise enzymes convey a 3D structure that:
  - Allows only specific substrates and cofactors to bind with the enzyme
  - Aligns the substrate with the reaction center of the enzyme
- The 3D enzyme structure and catalytic activity can be lost by exposing the enzyme to high temperatures, salinity, pH, and other extremes. These extremes “denature” the enzyme.
- Many enzymes have a reaction center that contains a metal cofactor, such as Mg, Mo, Cu, Fe, etc. Complex cofactors are called coenzymes, and if tightly bound, prosthetic group
- Regulatory (or allosteric) enzymes are affected by other compounds (modulators) that can either inhibit or activate an enzyme’s catalytic properties.

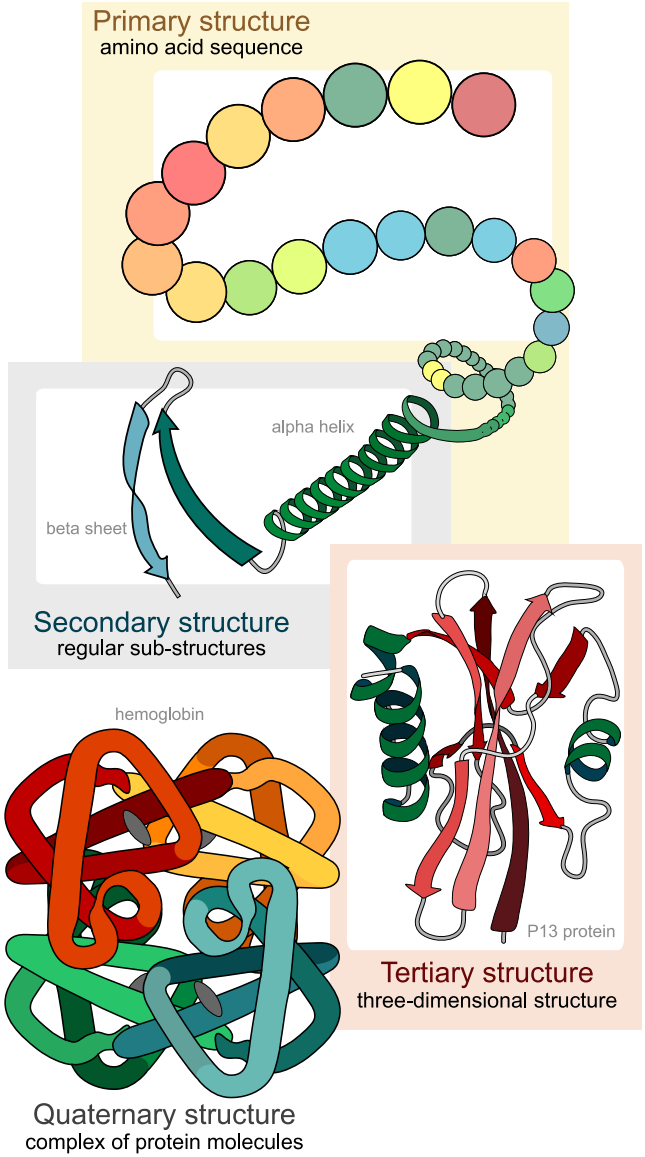


With modulator, enzyme can bind with substrate to produce product

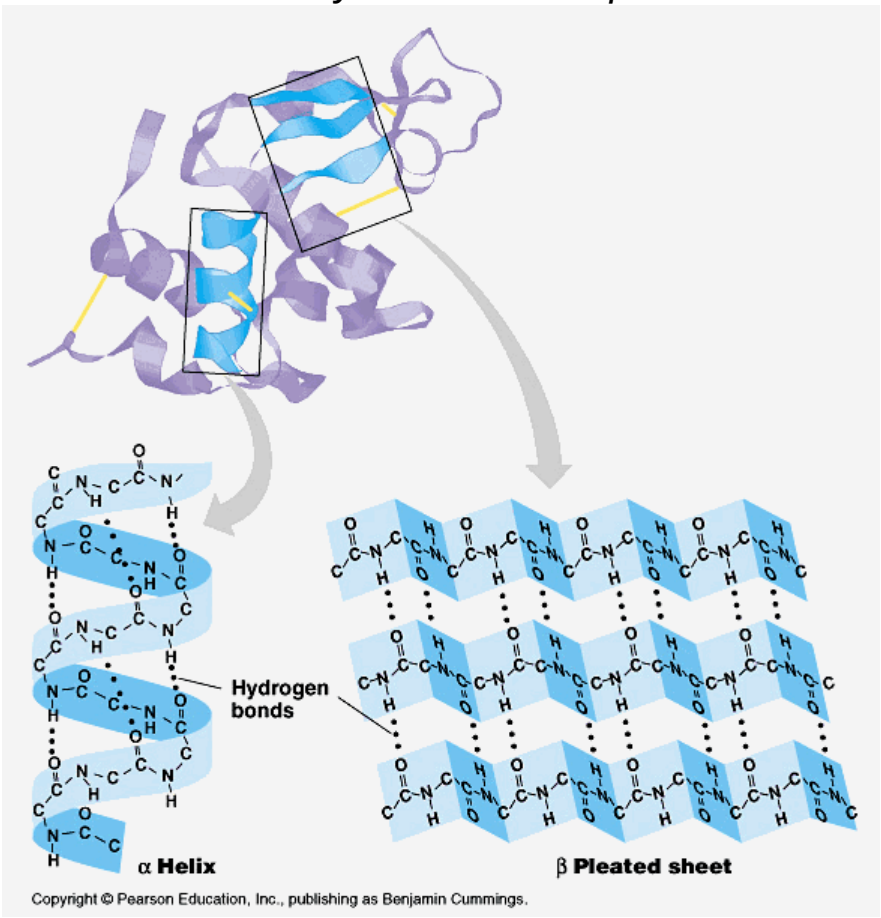


Without modulator, enzyme can not bind with substrate

Protein Structures



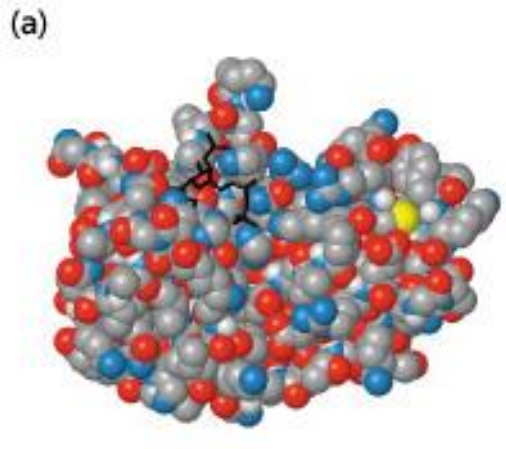
Secondary α-Helix and β-Sheets



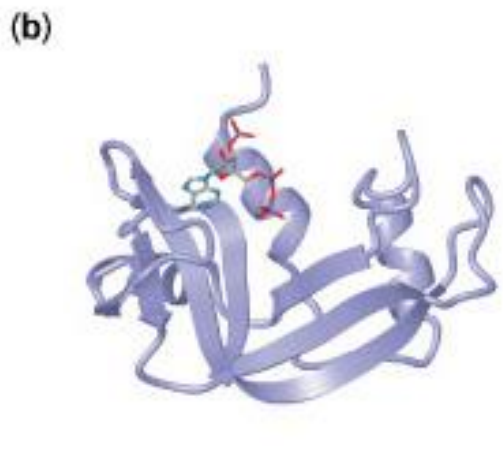
https://commons.wikimedia.org/w/index.php?curid=4945067

Bovine ribonuclease A

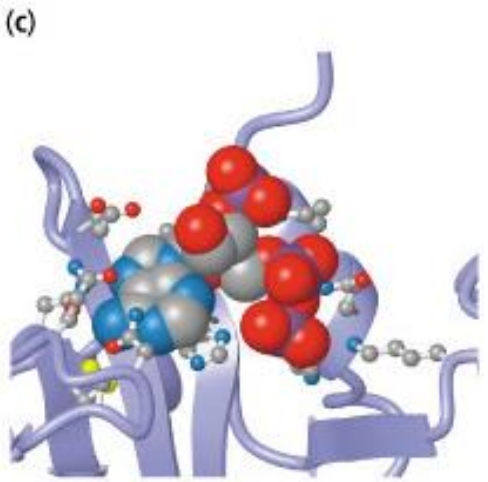
hydrolyzes RNA during digestion



space-filling model of RNAse A with a bound substrate



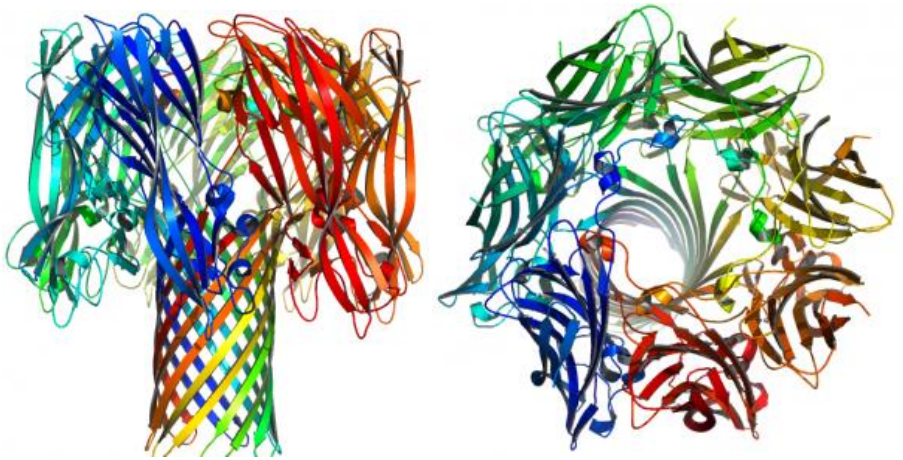
ribbon model of the protein backbone



substrate binding site

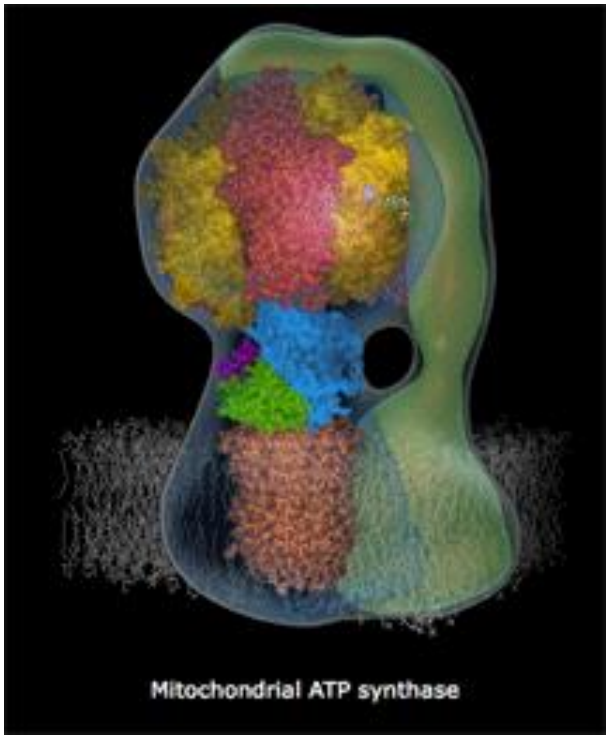
For molecular structures and imaging see
http://pdbwiki.org/
http://www.umass.edu/microbio/chime/top5.htm
http://rasmol.org/
http://polyview.cchmc.org/polyview3d.html
http://www.ncbi.nlm.nih.gov/Structure

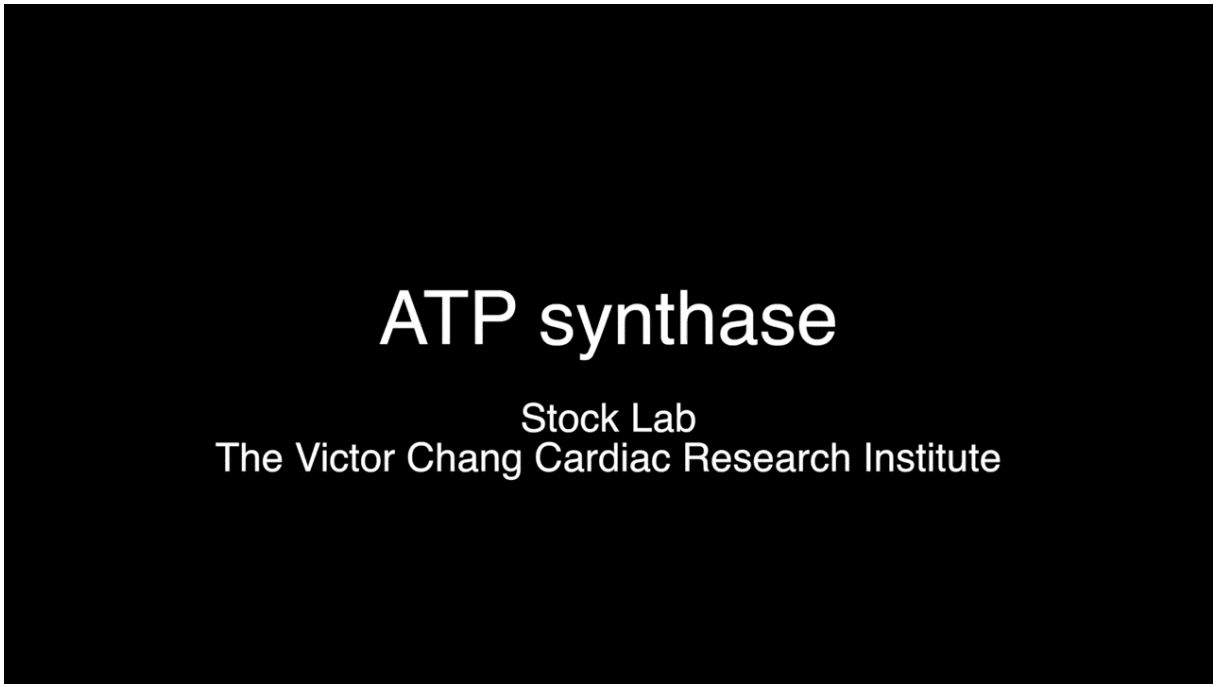
Foldit: https://fold.it/portal/ Crowdsource solns



Staphylococcus aureus porin protein

Mitochondrial ATP Synthase



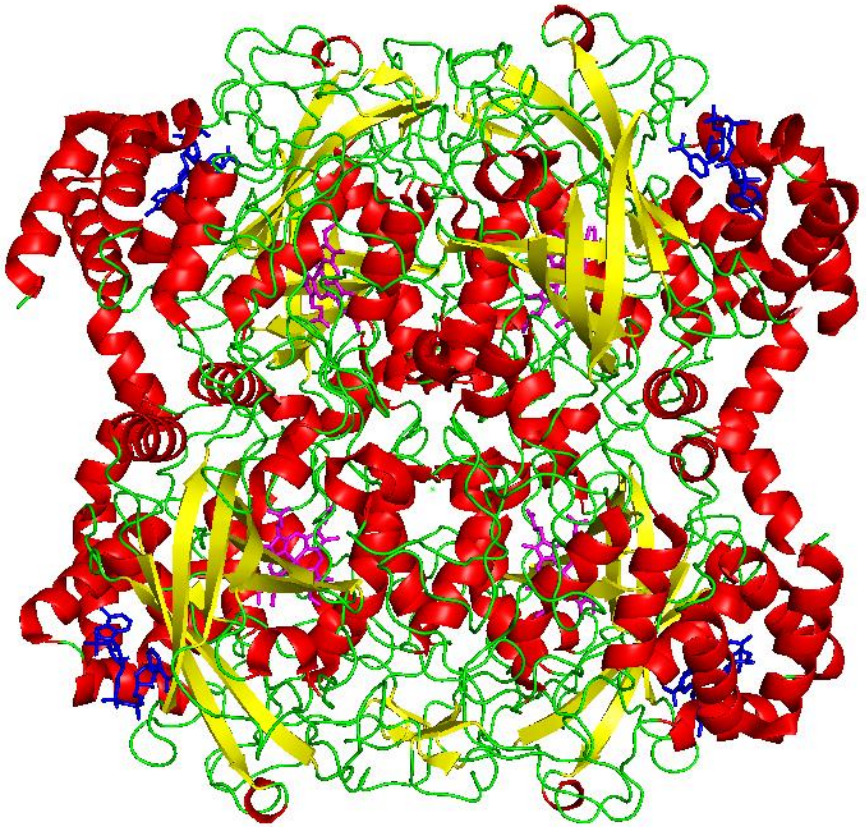


The central rotor turns in the direction shown about 150 times every second

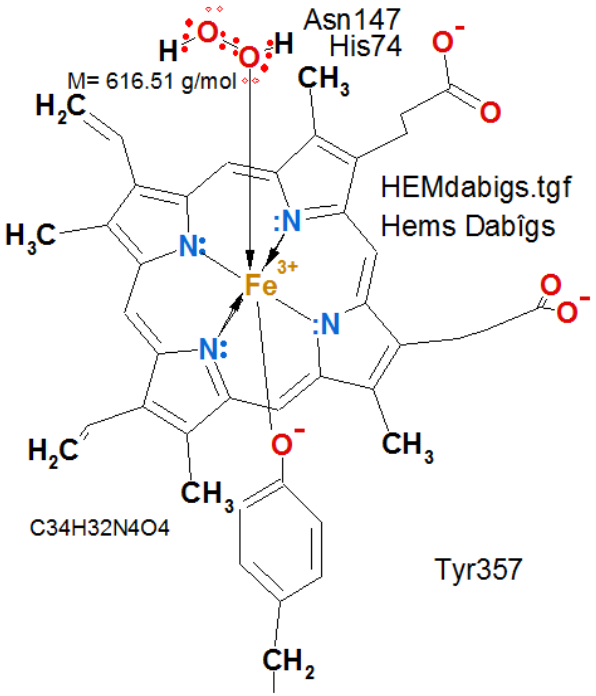
See: http://www.mrc-mbu.cam.ac.uk/projects/2245/atp-synthase
Good animation here: https://www.youtube.com/watch?v=b_cp8MsnZFA

Catalase

$$2H_2O_2 \rightarrow 2H_2O + O_2$$



Cofactor (prosthetic group)



http://genomics.unl.edu/RBC_EDU/cat.html

http://aris.gusc.lv/ChemFiles/catalaseKenyon/frames/hemetx.htm

Reaction Kinetics

- **Elementary Reactions**

Reaction	*Order*	*Rxn Rate*	*Units of k*
$A \rightarrow B$	First	V = k[A]	d^{-1}
$A + B \rightarrow C$	Second	V = k[A][B]	$d^{-1}M^{-1}$

- **Complex Reactions**

Observed:
$A \rightarrow F$

Propose mechanism consisting of elementary reactions:
$A \rightarrow B + C$
$B \rightarrow D$
$C \rightarrow E$
$D + E \rightarrow F$

- **Derive reaction kinetics**

$$\frac{d[F]}{dt} = V = k_4[D][E]$$

Need to solve for [D] and [E], etc so that given the concentration of A, the overall reaction rate can be determined.

Enzyme Kinetics

Mechanism

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES$$ Enzyme binds w/ substrate

$$ES \xrightarrow{k_2} E + P$$ Enzyme releases product

$$S \xrightarrow{E} P$$

Kinetic equations

$$\frac{d[P]}{dt} = V_0 = k_2[ES]$$

$$\frac{d[ES]}{dt} = k_1[E][S] - k_{-1}[ES] - k_2[ES]$$

$$\frac{d[S]}{dt} = k_1[E][S] - k_{-1}[ES]$$

Analytical solution is not possible with above equations; however,

Steady state assumption turns out to be good approximation

$$\frac{d[ES]}{dt} \cong 0$$

And use:

$$[E_T] = [E] + [ES]$$

Derivation

$$\frac{d[ES]}{dt} = k_1[E][S] - k_{-1}[ES] - k_2[ES] \overset{SSA}{\Rightarrow} k_1[E][S] = (k_{-1} + k_2)[ES]$$

$$[E] = [E_T] - [ES]$$

$$k_1([E_T] - [ES])[S] = (k_{-1} + k_2)[ES]$$

$$[ES] = \frac{k_1[E_T][S]}{k_{-1} + k_2 + k_1[S]} = \frac{[E_T][S]}{\frac{k_{-1} + k_2}{k_1} + [S]}$$

$$v_o = k_2[ES] = \frac{k_2[E_T][S]}{\frac{k_{-1} + k_2}{k_1} + [S]}$$

Michaelis-Menten Kinetics

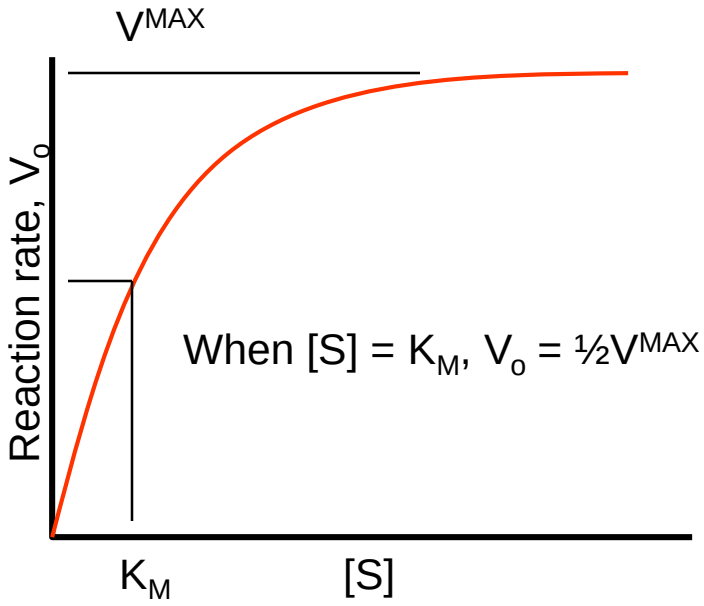
The four kinetic equations can be solved to give:

$$V_o = \frac{V^{MAX}[S]}{K_M + [S]}$$

where

$V^{MAX} = k_2[E_T]$ Maximum reaction rate

$K_M = \frac{k_{-1} + k_2}{k_1}$ Michaelis-Menten constant, or half saturation constant



Asymptotes

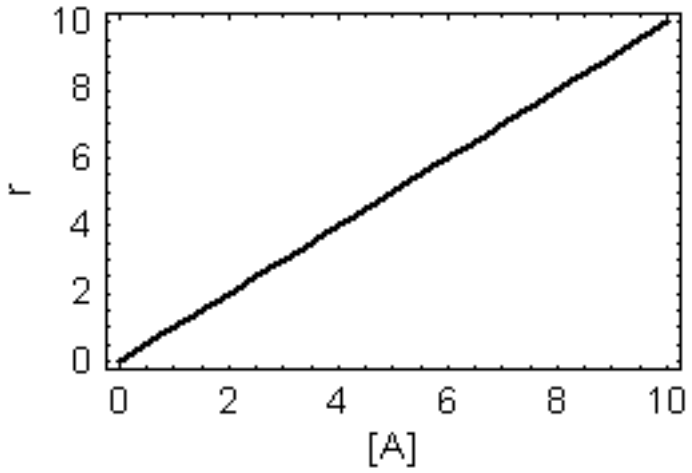
As $[S] \to \infty$; $V_o \to V^{MAX}$

As $[S] \to 0$; $V_o \to \frac{V^{MAX}}{K_M}[S] = k[S]$

Michaelis-Menten versus 1st order kinetics

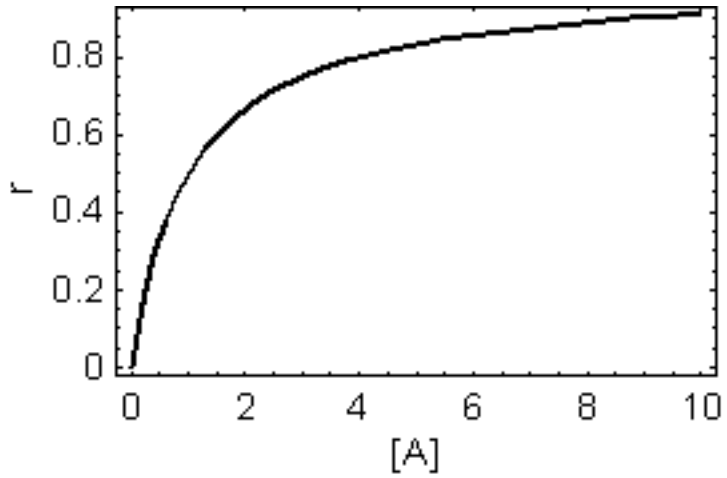
First Order Kinetics

$r = k_1[A]$; $k_1 = 1$



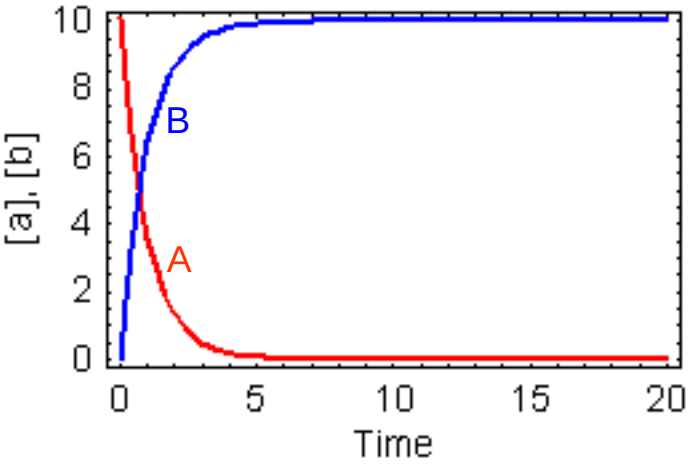
Michaelis-Menten Kinetics

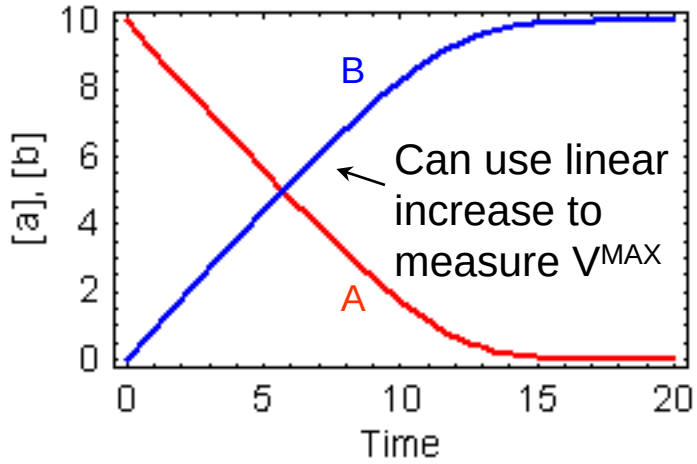
$$r = \frac{V^{MAX}[A]}{K_M + [A]};\ V^{MAX} = 1,\ K_M = 1$$



$A \rightarrow B$

$$\frac{d[A]}{dt} = -r; \quad \frac{d[B]}{dt} = r$$

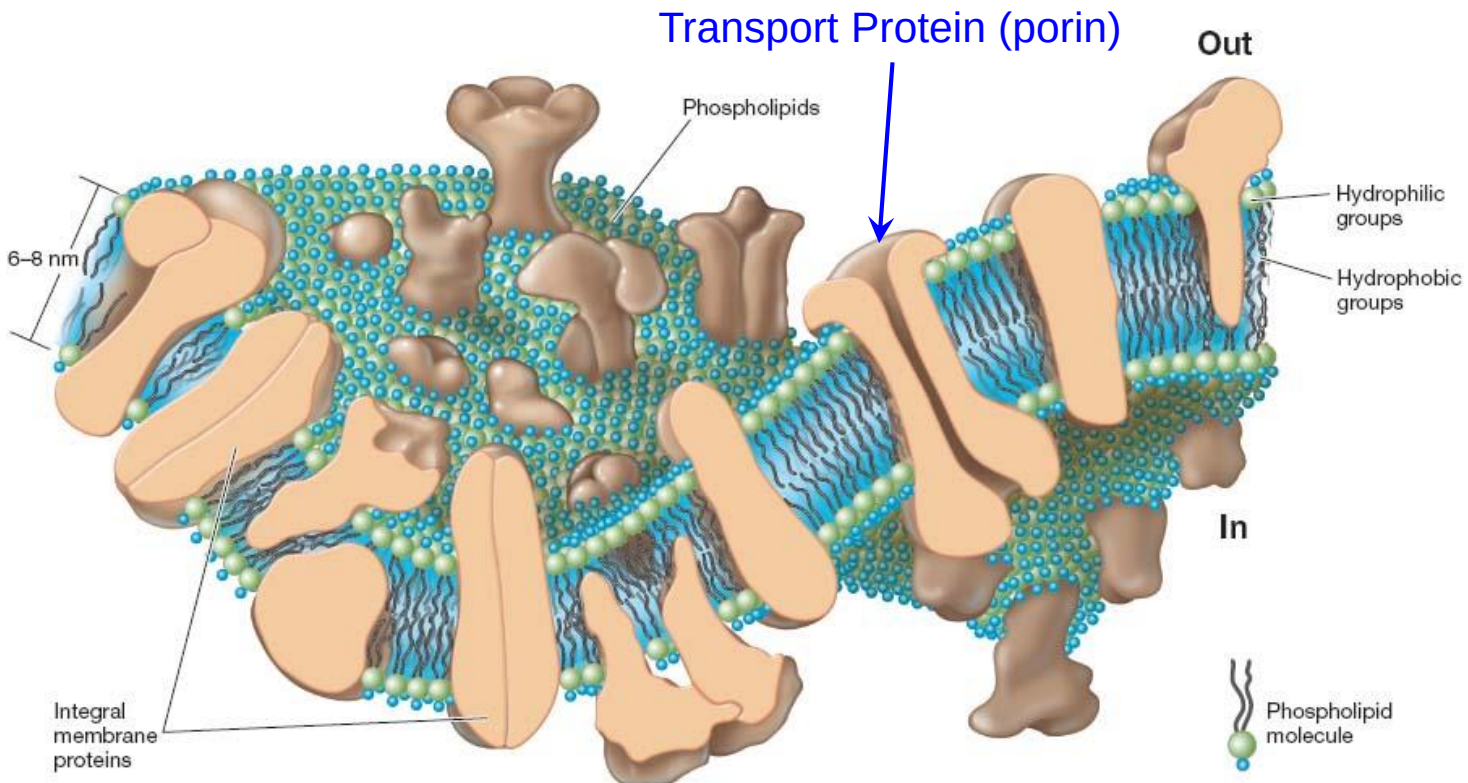




For Michaelis-Menten kinetics, the increase in [B] and the decrease in [A] occurs linearly while [A] >> K_M

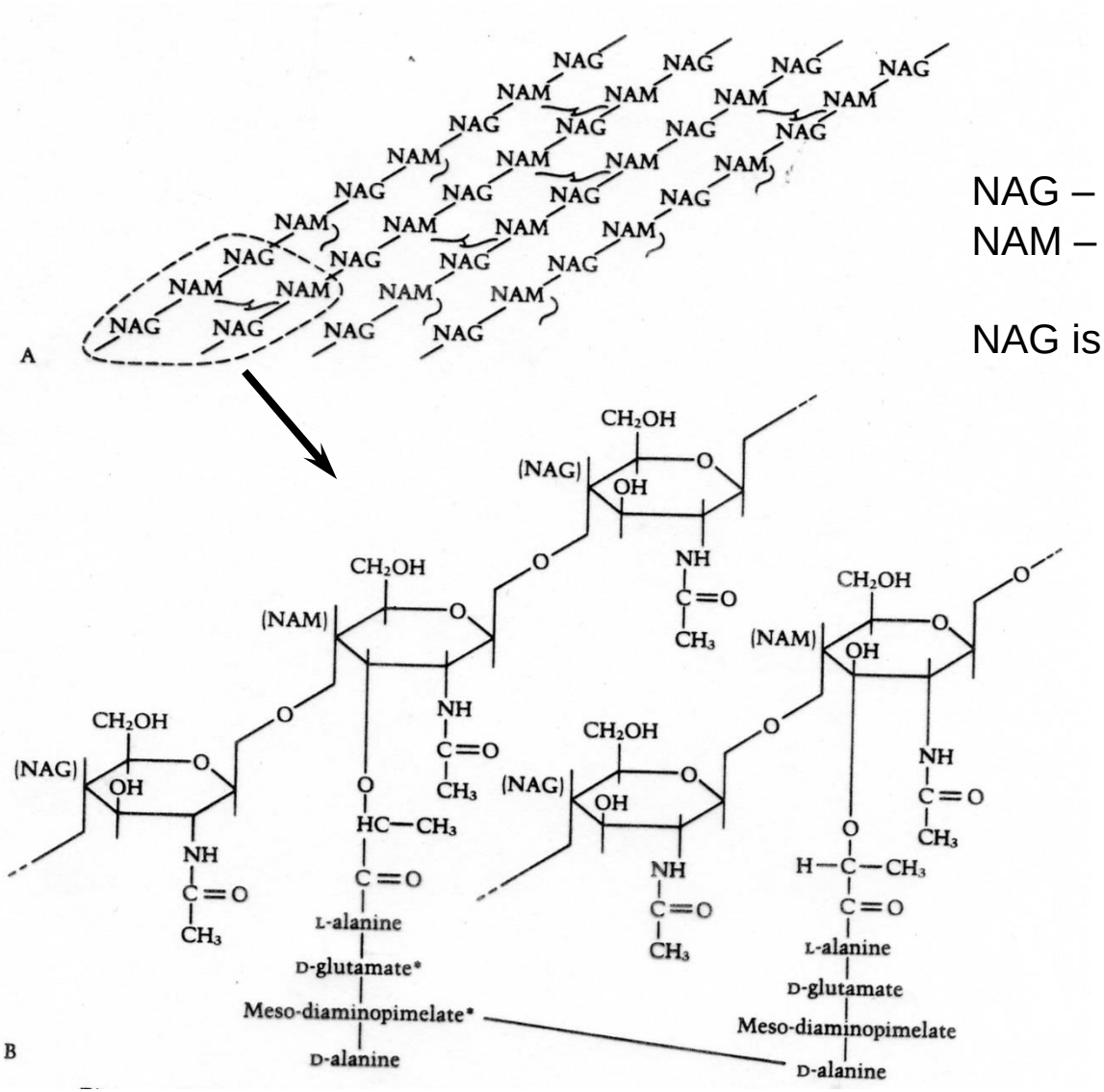
Transport across the cell membrane

- The concentration of substrates outside the cell are usually at low concentration, i.e. nM
- The concentration of substrates inside the cell are usual at high concentrations, i.e. mM
- Consequently, the cell must actively transport material across the cell membrane.
- Special proteins embedded in the cell wall and membrane are responsible for transporting material into and out of the cell.
- **These transport systems only operate on relative small molecules, i.e. < 1000 MW**



See Brock, pp. 73-75
Sec. 4.4

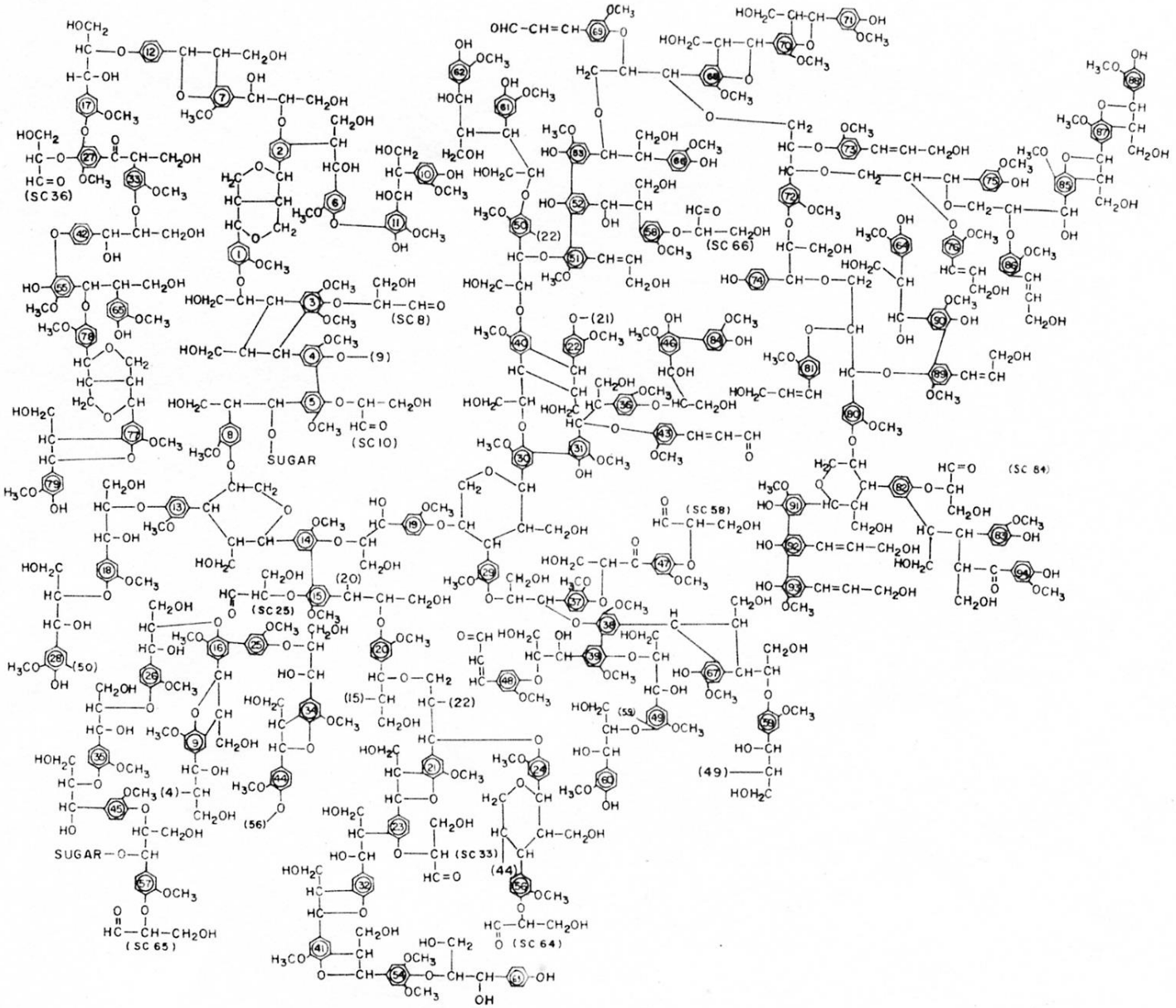
Peptidoglycan Example (*E. coli*)



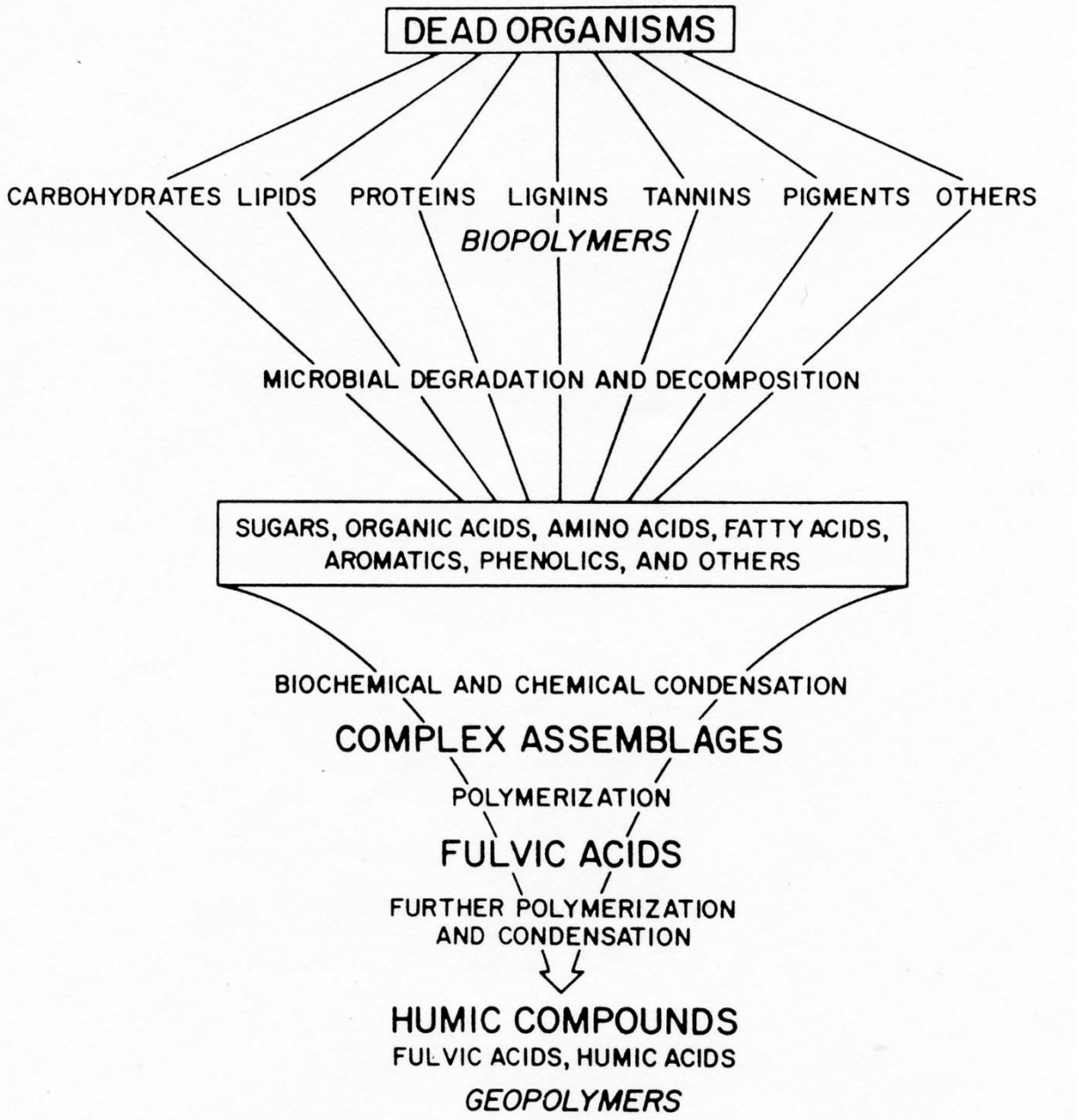
NAG – N-acetylglucosamine
NAM – N-acetylmuramic acid

NAG is also the monomer of chitin

Possible Lignin Structure



Diagenetic Reactions



Bacterial Substrates

- All organisms are comprise of mostly polymeric material: protein, cellulose, starch, lipids, peptidoglycans, lignin, RNA, DNA, etc.
- Consequently, dead organic material available for bacterial consumption is mostly large polymeric material with high molecular weights.
- **Large polymeric compounds can not be transported across the cell wall.**
- As organic material is exposed to environmental factors, such as ultraviolet light, absorption onto minerals (clay, etc) and bacterial degradation, the organic material becomes even more amorphous.

Problem: monomers exist at low concentration and make up only a small percent of the extracellular POM+DOM pool.

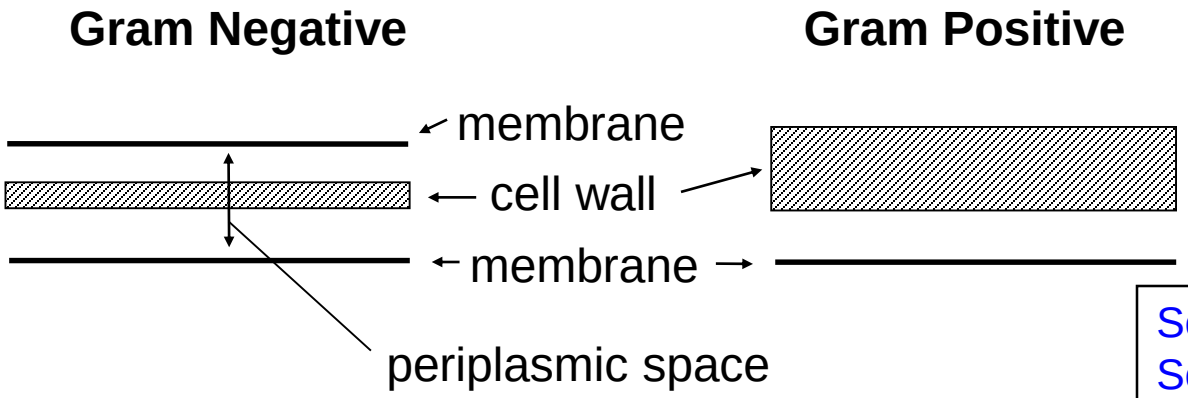
How do bacteria breakdown and consume the large polymeric material?

Extracellular Enzymes

In order to breakdown large polymeric organic material into small monomers, bacteria produce extracellular and ectoenzymes.

- Extracellular Enzymes: Excreted from cell and exist in solution in free form.
- Ectoenzymes: Bound to cell surface, but can attack extracellular substrates.

Both types of enzymes are produced by both Gram negative and Gram positive bacteria

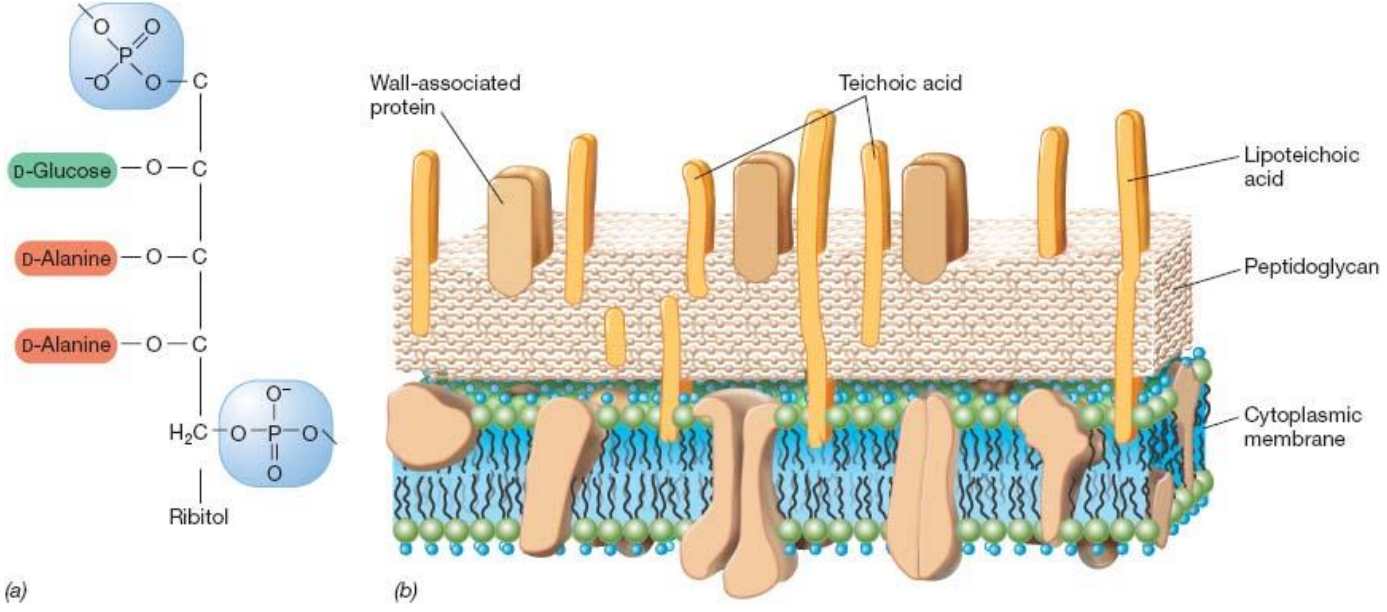


See Brock, pp. 78-84
Sec. 4.6-4.7

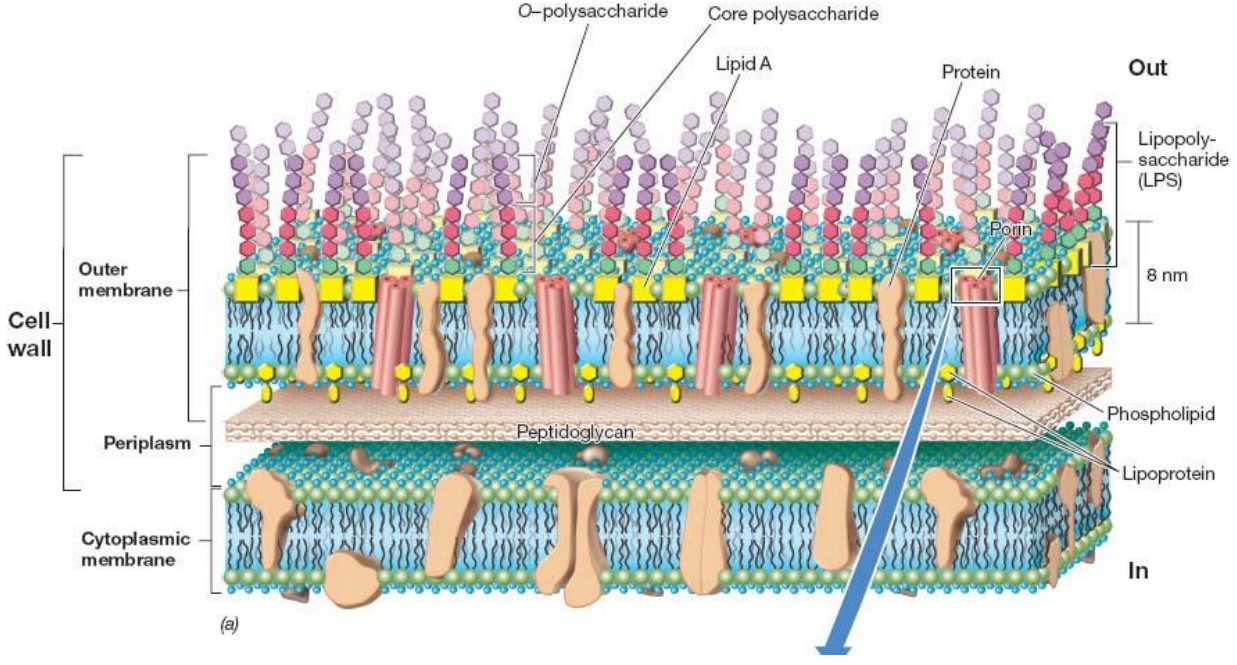
Bacterial growth may often be limited by the hydrolysis rate of extracellular macromolecules (area of current research).

Gram Positive & Negative Cell Wall Diagrams

Gram Positive



Gram Negative



Enzyme Assay

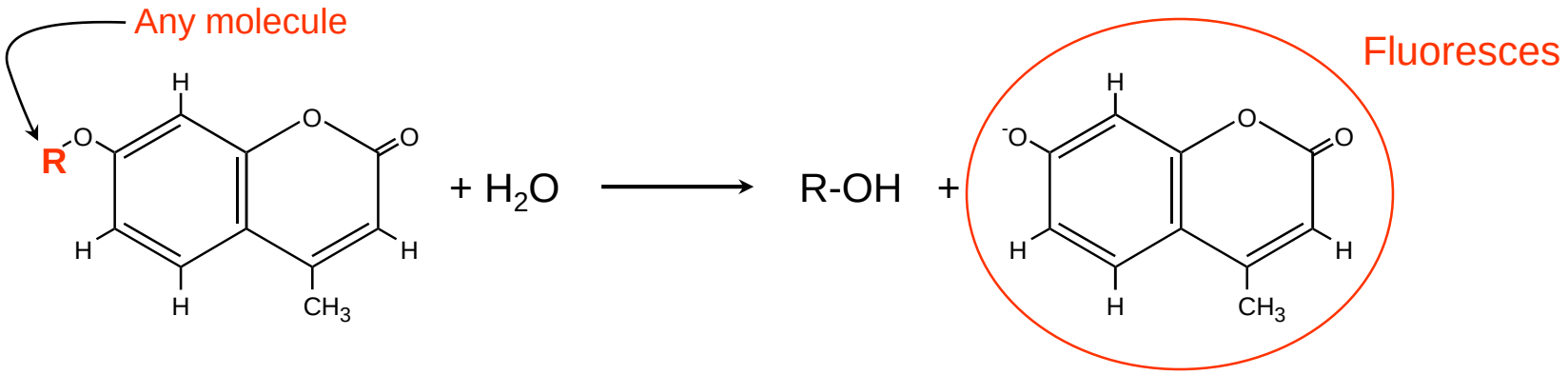
- Extracellular and ectoenzymes catalyze hydrolysis reactions, which are exoergic, so do not require an energy source, such as ATP, to proceed:

  … -A-A-A-A-… + H_2O → …-A-A-OH + H-A-A-…

- Because of the low concentrations of the substrates, it is not practical to measure the decrease in concentration of the natural substrates.
- Fluorogenic substrates are used as analogs to the true substrates.

MUF-R: 4-methylumbelliferyl-R

MUF: 4-methylumbelliferyl

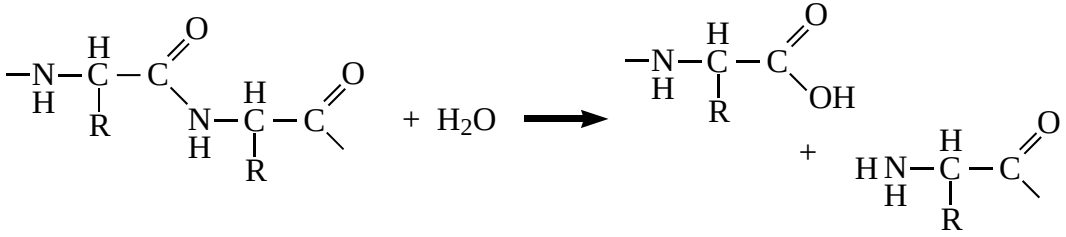


MUF fluoresces provided nothing is bound to O^-. At low pH, MUF will become MUF-H and will not fluoresce.

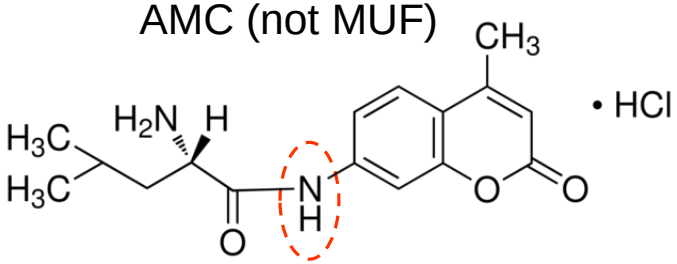
- By using different molecules bound to MUF (i.e., -R), different enzymes can be assayed.

MUF Enzyme Assays

- **Endopeptidase**



AMC (not MUF)



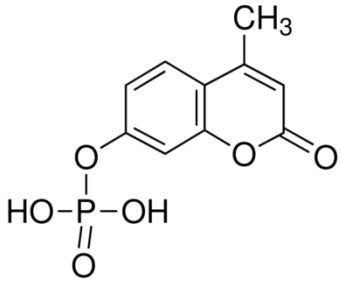
Note, an AMC standard should be used instead of MUF, but they have similar fluorescence, so for class we will use MUF standard.

Assay substrate: L-Leucine-7-amido-4-methylcoumarin hydrochloride

- **Phosphatase**

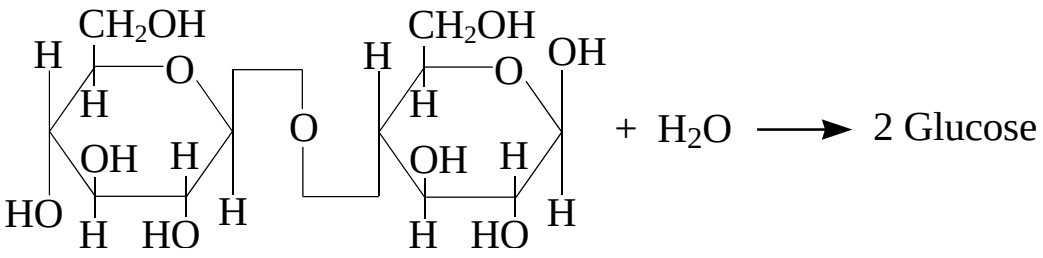
$$R\text{-}O\text{-}PO_3^{2-} + H_2O \longrightarrow R\text{-}OH + HPO_4^{2-}$$

Assay substrate: MUF-phosphate

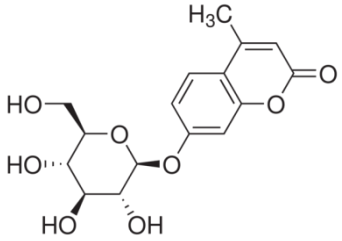


More MUF Enzyme Assays

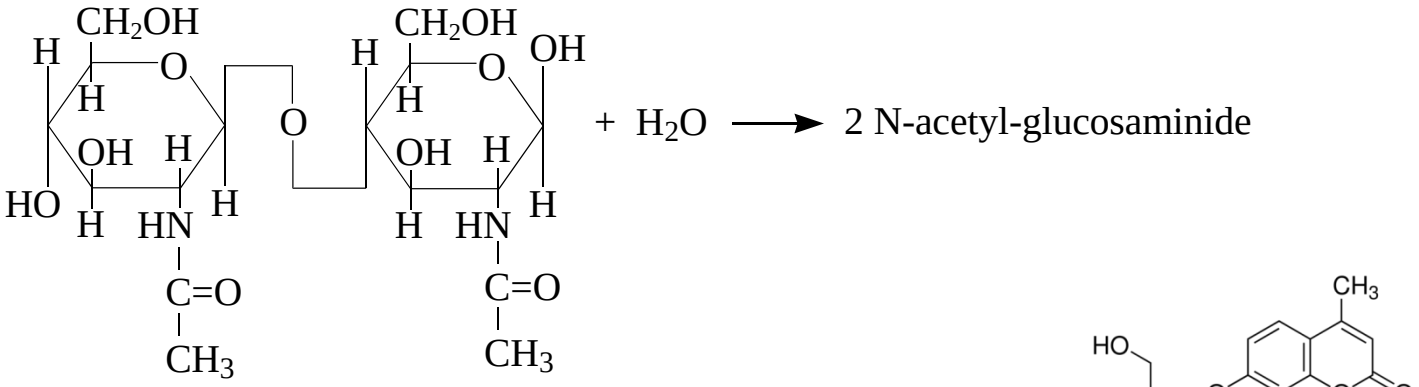
- **β-1,4-glucosidase (cellobiase)**



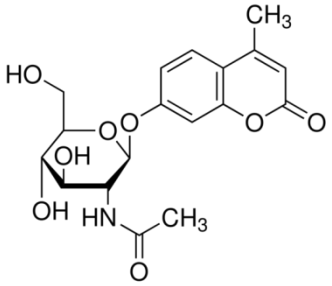
Assay substrate: MUF-β-D-glucopyranoside



- **N-β-D-acetyl-glucosaminidase (Chitobiase)**

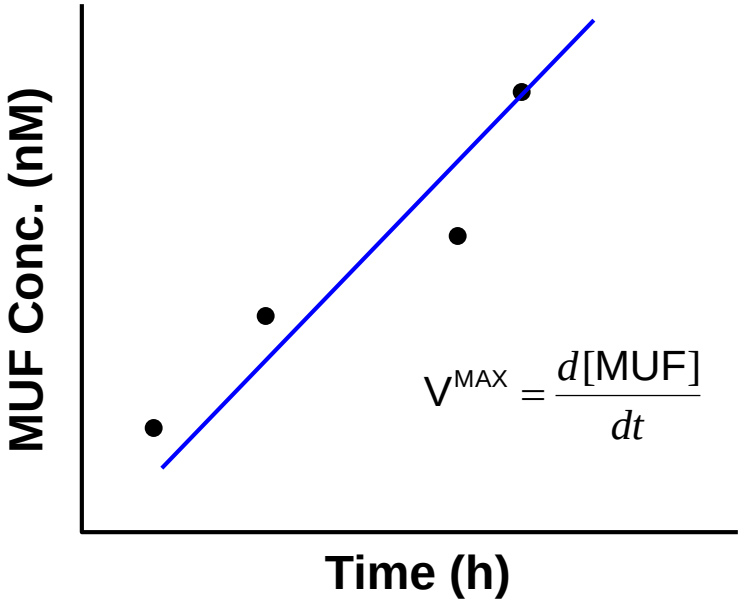


Assay substrate: MUF-N-β-D-acetyl-glucosaminide



Eco- and Extracellular Enzyme Assay

- Introduce MUF substrate for enzyme to be assayed
- Measure accumulation of free MUF with fluorometer over time.
- Plot MUF concentration versus time (make sure to account for dilution).
- Determine slope of line that best fits the data. This slope is V^{MAX}



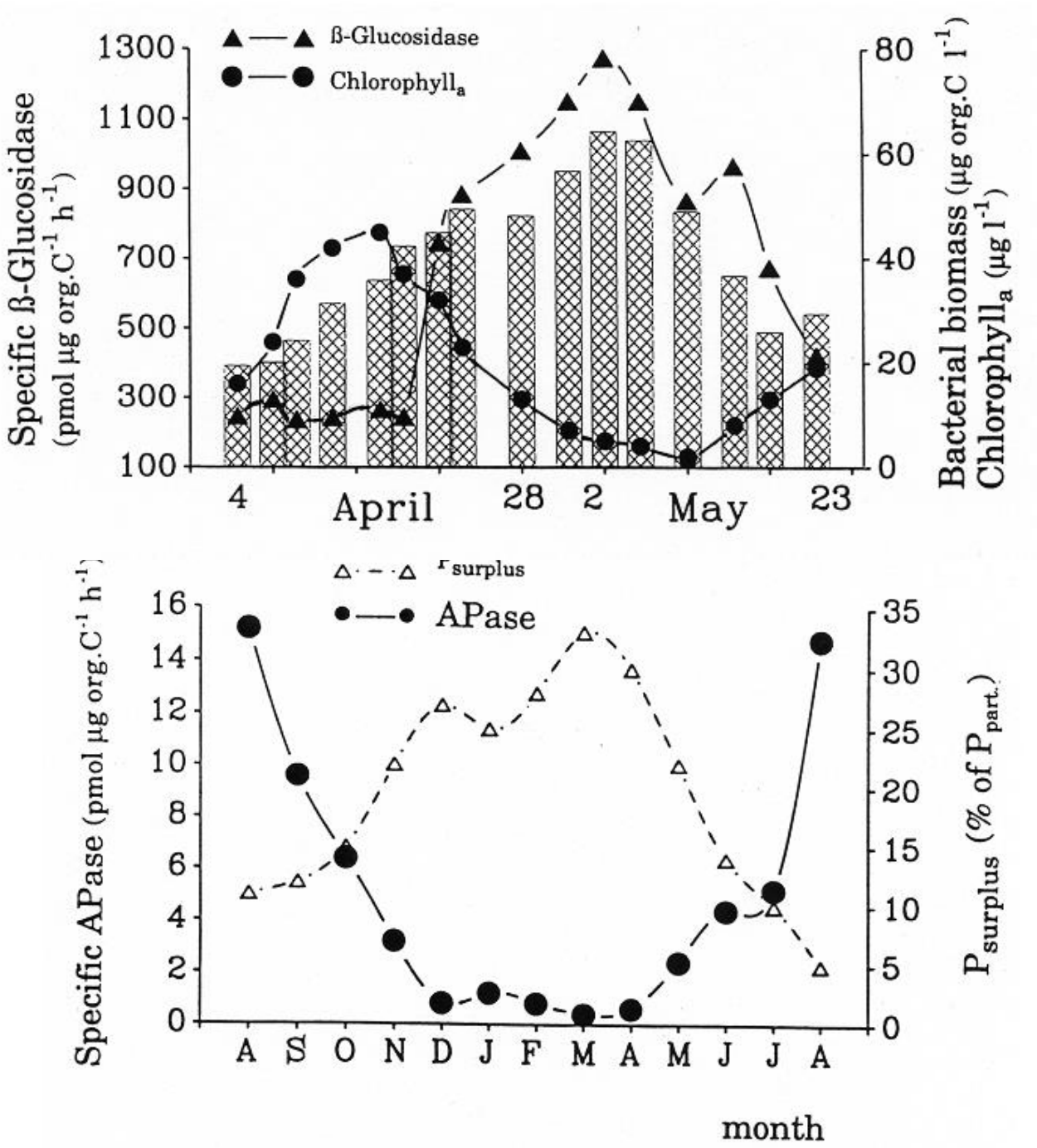
Enzymes Assayed:

- Chitobiase
- Phosphatase
- Endopeptidase
- Cellobiase

How might V^{MAX} be used to determine the state of an ecosystem?

Note, the enzyme activity measured is not the activity that occurs in the natural environment! Why?

Example Applications



From: Chróst, R.J. (1991) Microbial enzymes in aquatic environments.