Enzymes III

Regulation

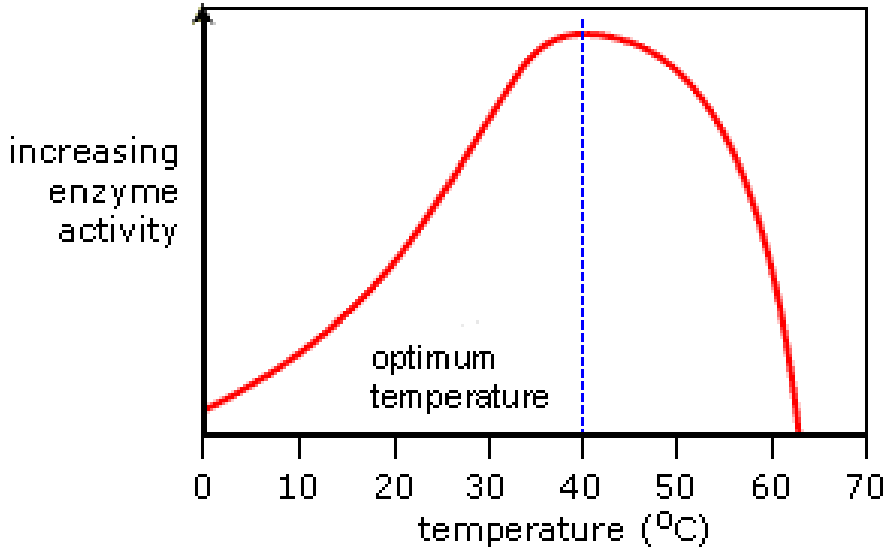
Summer semester, 2023

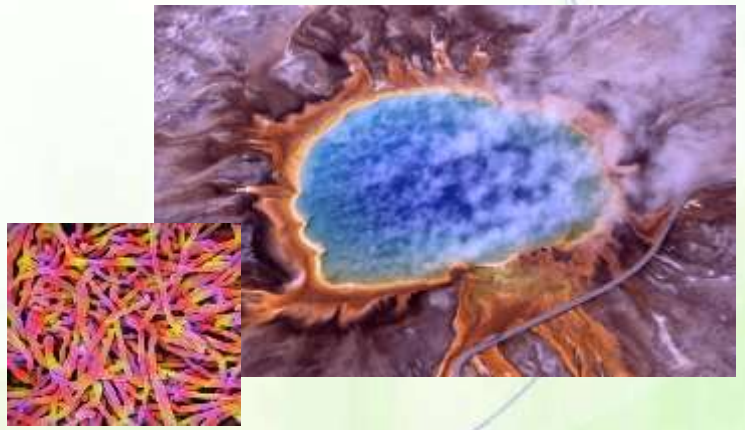
Mechanisms of regulation

- Non-specific regulation (temperature, pH, diffusion, and expression)
  - Localization (compartmentalization and complexing of enzymes)
  - Expression of isoenzymes
- Regulation of enzymatic activity
  - Inhibitors
  - Conformational changes
    - Modulators
    - Reversible covalent modification
    - Irreversible covalent modification
    - Allostery

Temperature

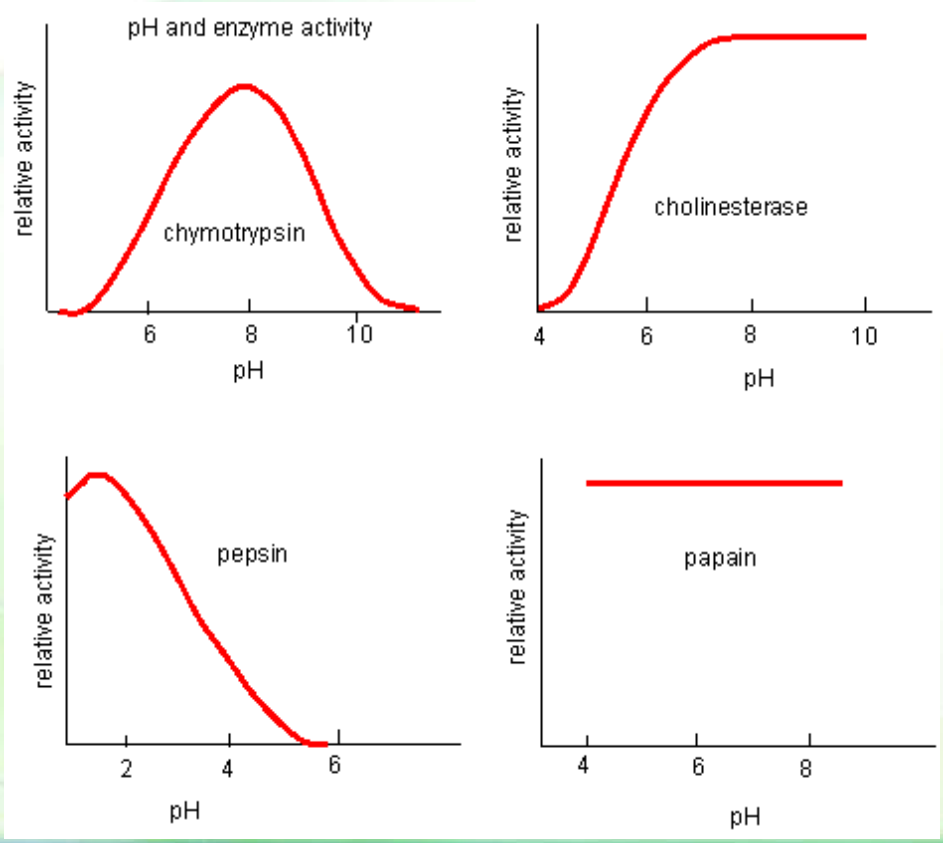
- Reaction rates increase with temperature due to increased kinetic energy of the molecules resulting in more collisions between enzymes and substrates.
- However, high temperatures lead to protein denaturation.
- Each enzyme has an optimal temperature.
- For thermophilic bacteria, the optimal temperature is as high as 65°C.





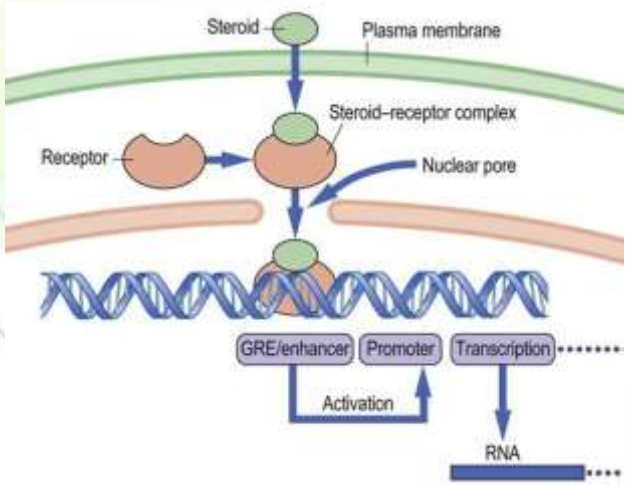
pH

- pH alters the protonation state of the substrate and/or the enzyme and, hence, their binding.
- The effect of pH is enzyme-dependent.



Regulation of enzyme amount

- Three mechanisms:
  - Enzyme synthesis at the gene level
  - Enzyme degradation by proteases
  - Synthesis of isozymes

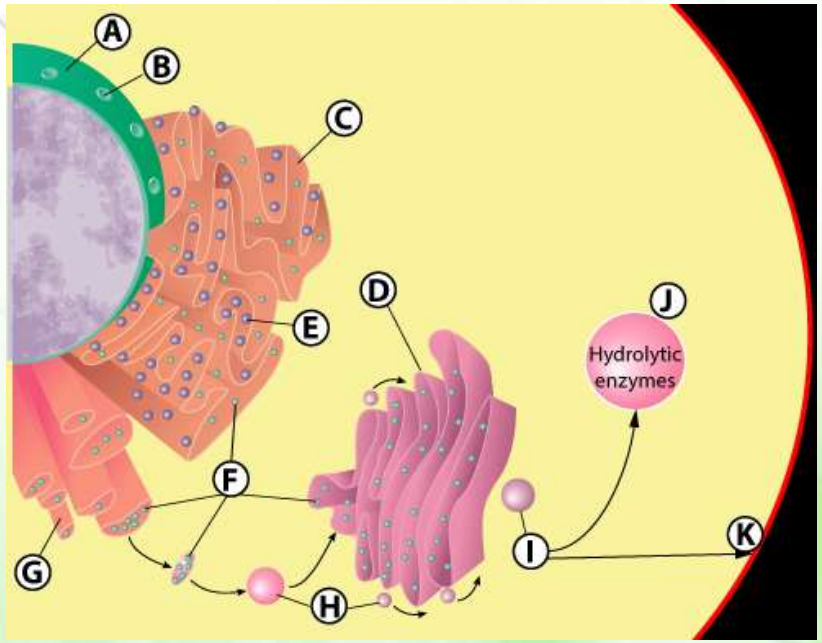


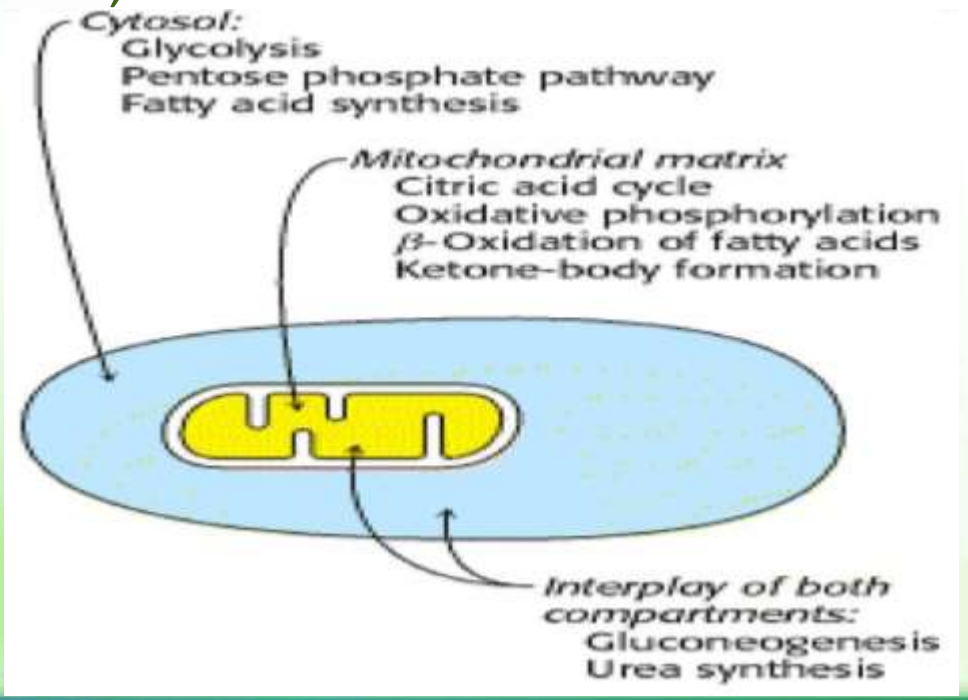
- They are comparatively slow mechanisms for regulating enzyme concentration (hours-weeks).

Enzyme	Half-life (days)
Catalase	1.4 days
Glucokinase	1.2 days
Lactate dehydrogenase	
LDH1 (heart)	1.6
LDH5 (liver)	16
LDH5 (muscle)	31

Compartmentalization

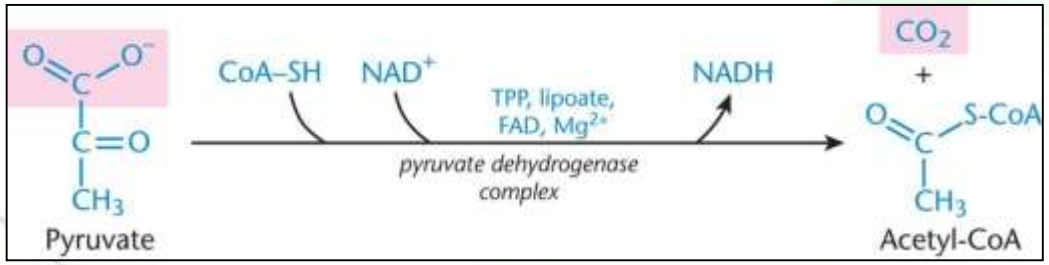
- Compartmentalization reduces the area of diffusion of both enzyme and substrate increasing the probability that they collide.
  - Example 1: lysosomal enzymes
  - Example 2: fatty acid metabolism
    - Synthesis occurs in cytosol, whereas break-sown is mitochondrial.

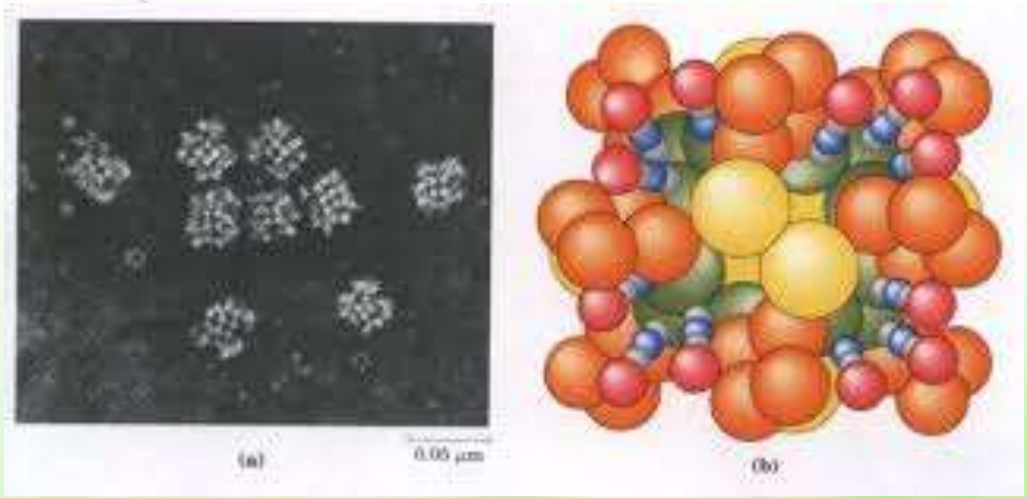


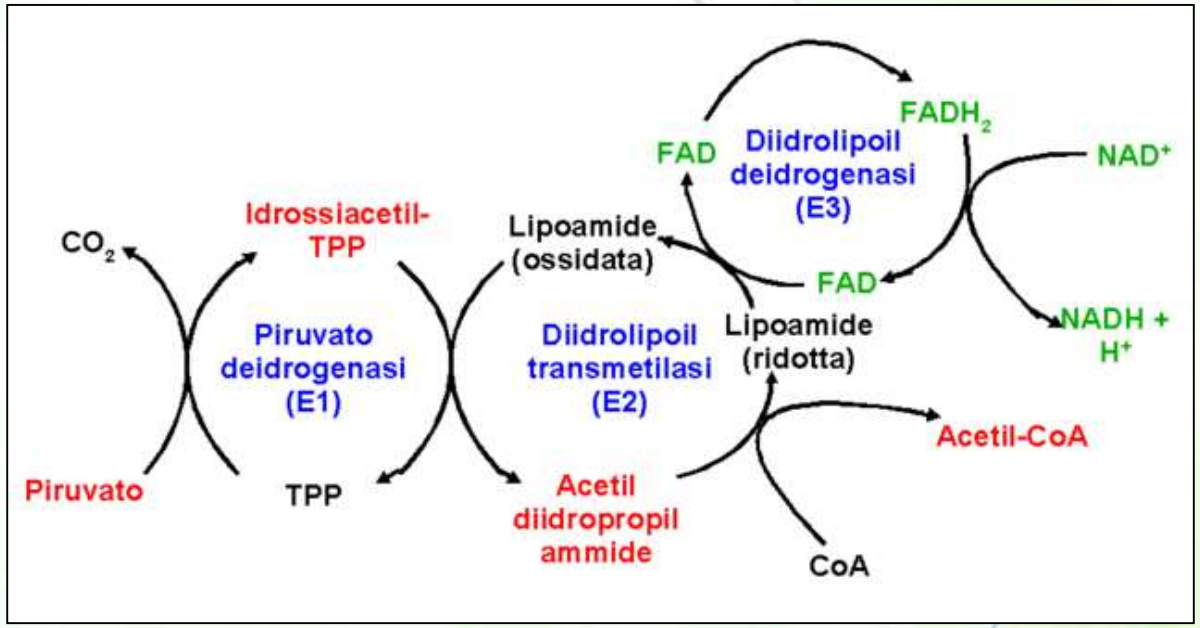


Enzyme complexing

- Formation of a complex of multiple enzymes also reduces diffusion.
- Example: Pyruvate dehydrogenase (mitochondria) is composed of 3 enzymes: decarboxylation, oxidation, & transfer of the acyl group to CoA.

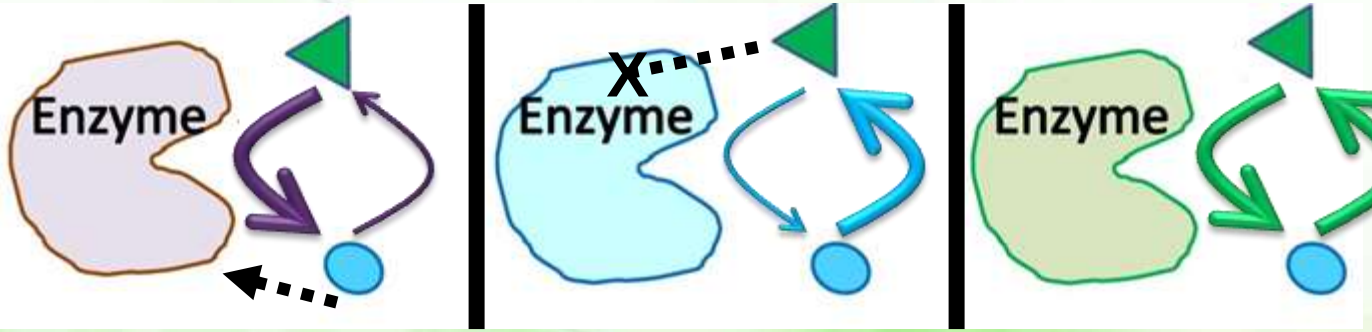


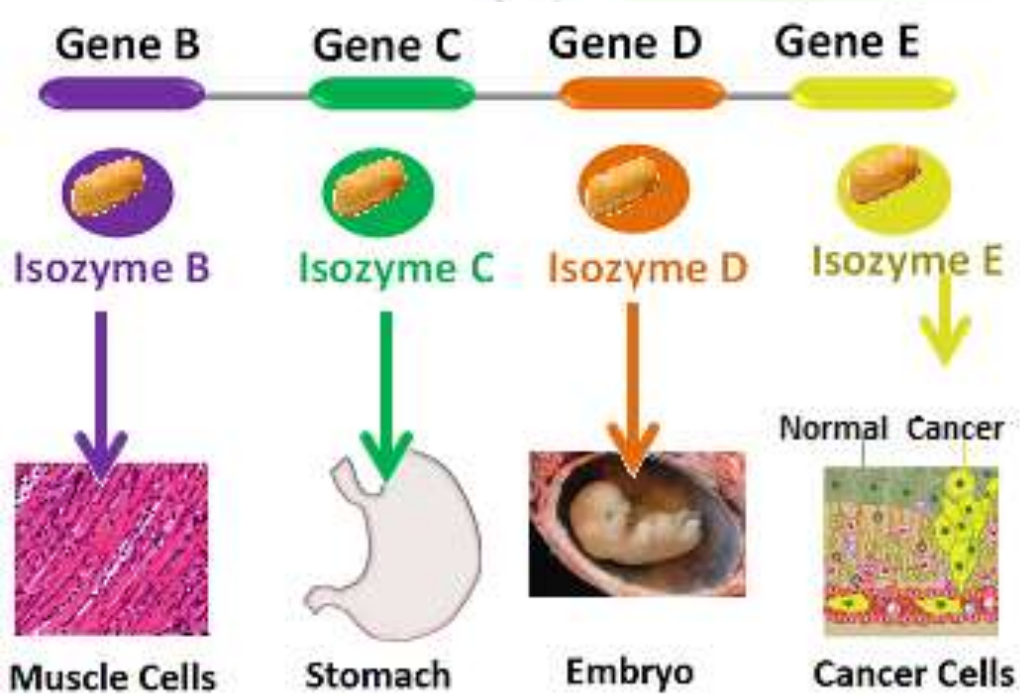




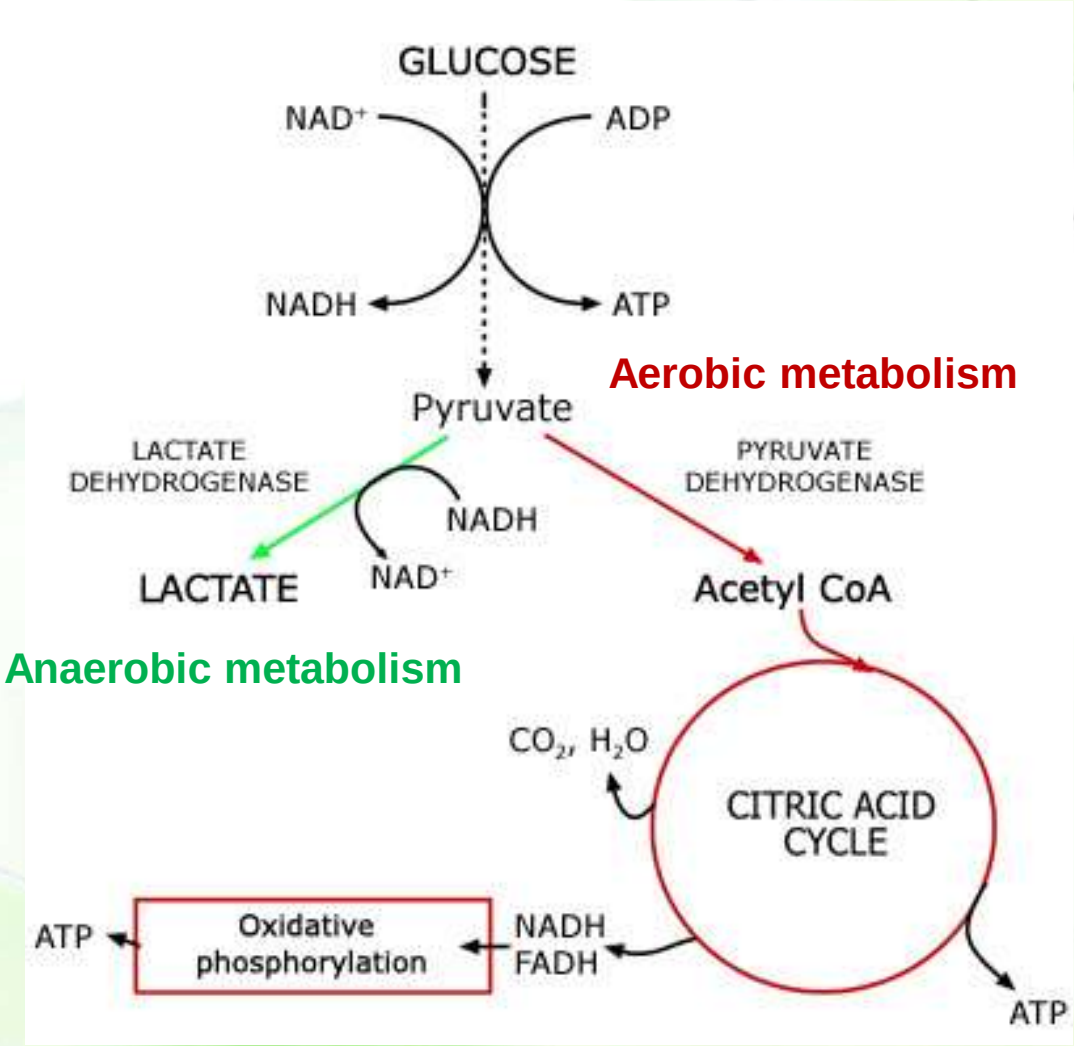
Isoenzymes (isozymes)

- Isoenzymes are enzymes that can act on the same substrate(s) producing the same product(s).
- They are produced by different genes that vary only slightly.
- Often, various isozymes are present in different tissues of the body.
- They can be regulated differently .
- They can have different catalytic activities.



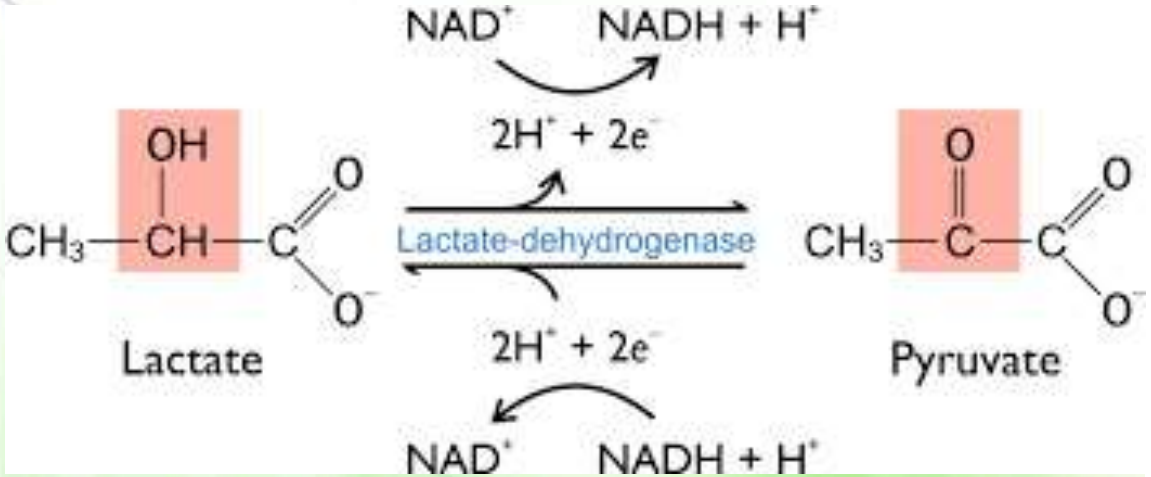


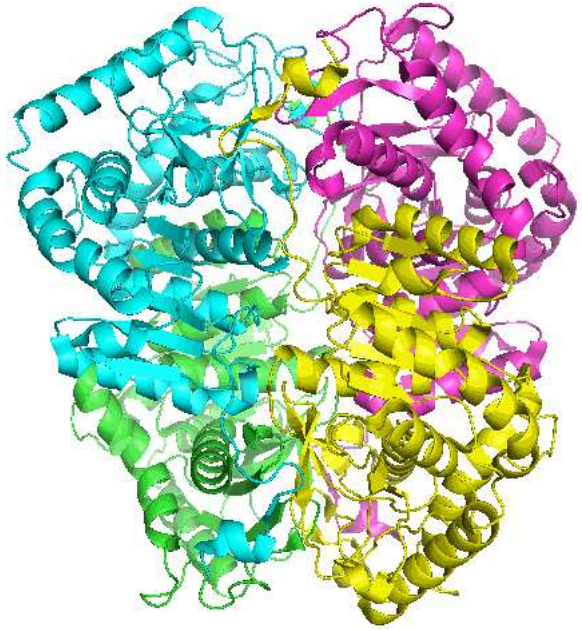
Aerobic vs. anaerobic metabolism

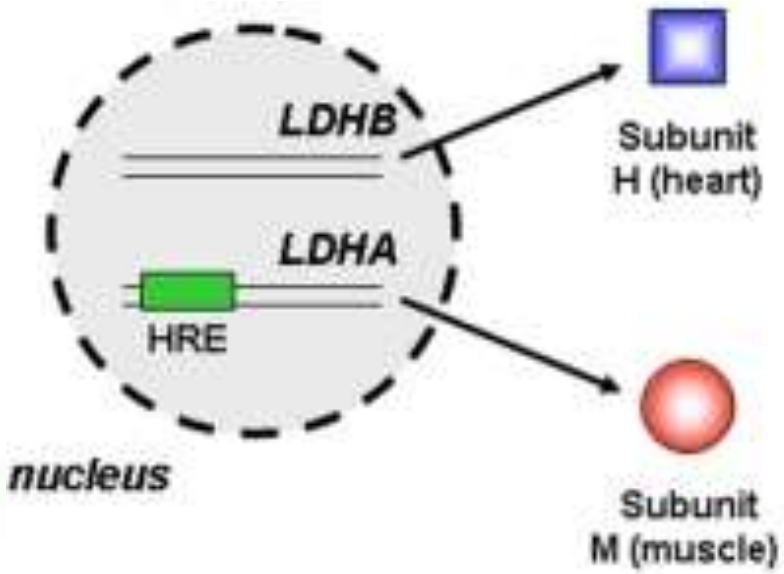


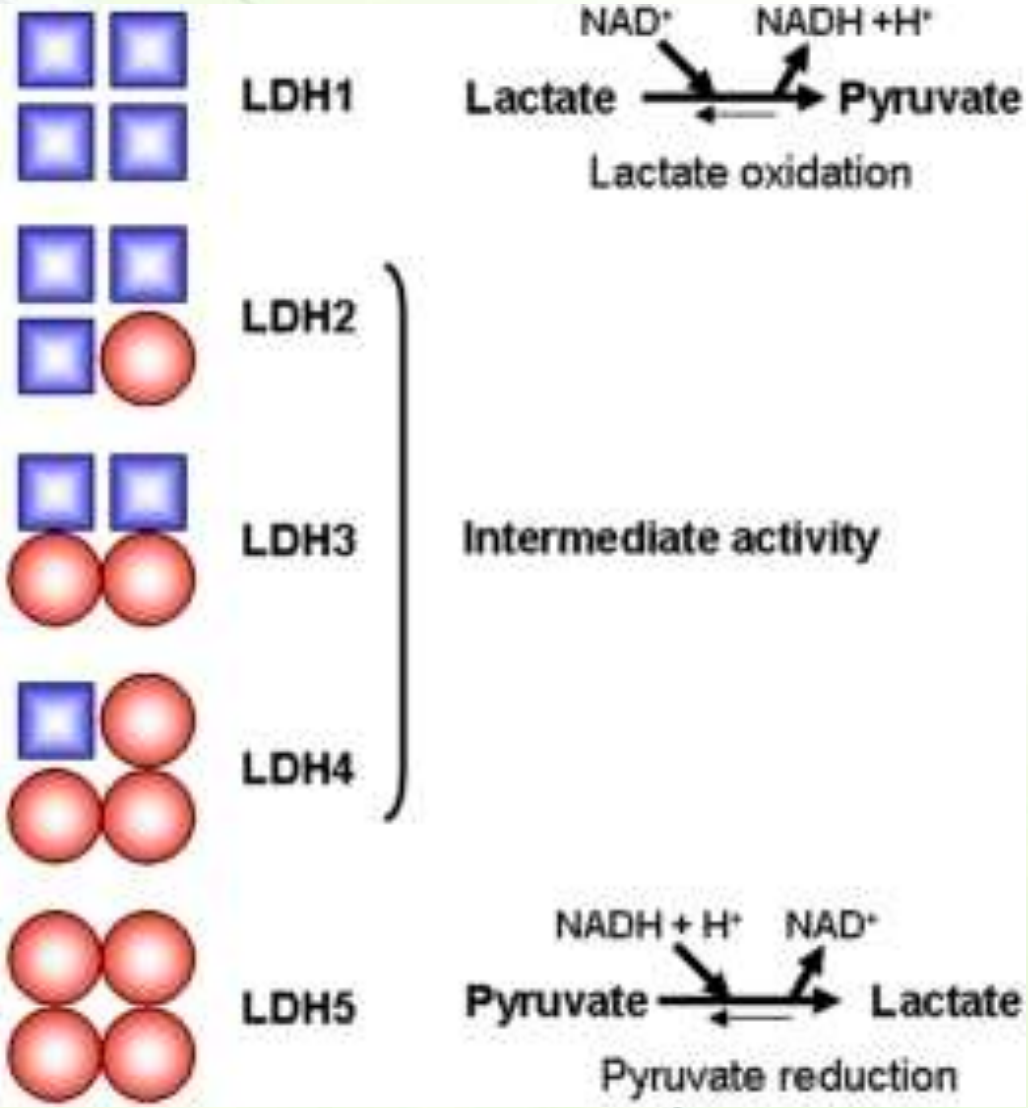
Lactate dehydrogenases (LDH)

- LDH is a tetrameric enzyme composed of a combination of one or two protein subunits: H (heart) and M (skeletal muscle).
- These subunits combine in various ways leading to 5 distinct isozymes leading to 5 distinct isozymes (LDH1-5) with different combinations of the M and H subunits.
- The all H isozyme is characteristic of that from heart tissue, and the all M isozyme is typically found in skeletal muscle and liver.



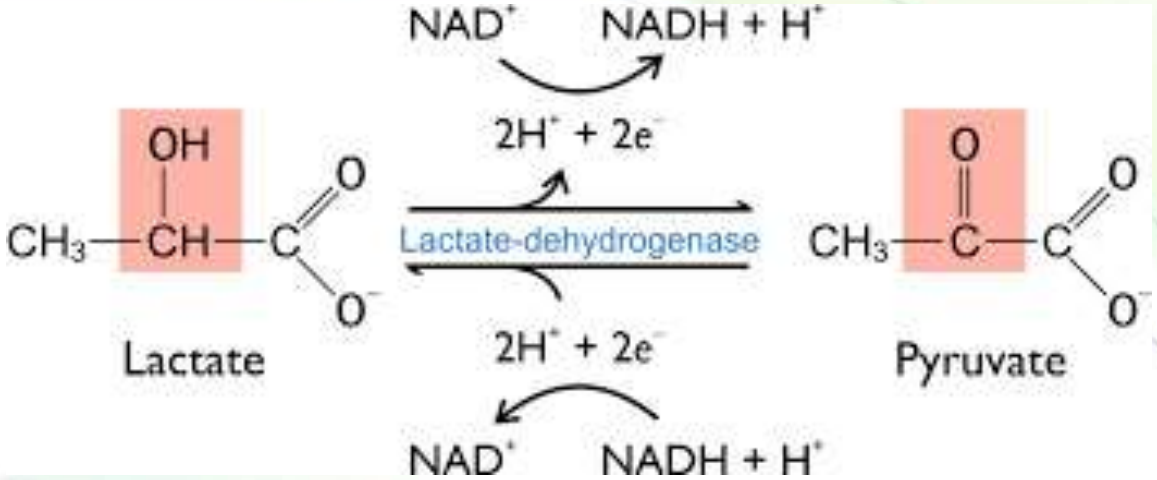






- Although the five isoforms catalyze the same reaction, they differ in their primary structure (slightly), kinetic properties, tissue distribution, affinity to the substrate, regulation, and isoelectric point.
- The M subunit has a net charge of (-6) and higher affinity towards pyruvate, thus converting pyruvate to lactate (and NADH to NAD+).
- The H subunit has a net charge of (+1) and a higher affinity towards lactate, resulting in a preferential conversion of lactate to pyruvate (and NAD+ to NADH).

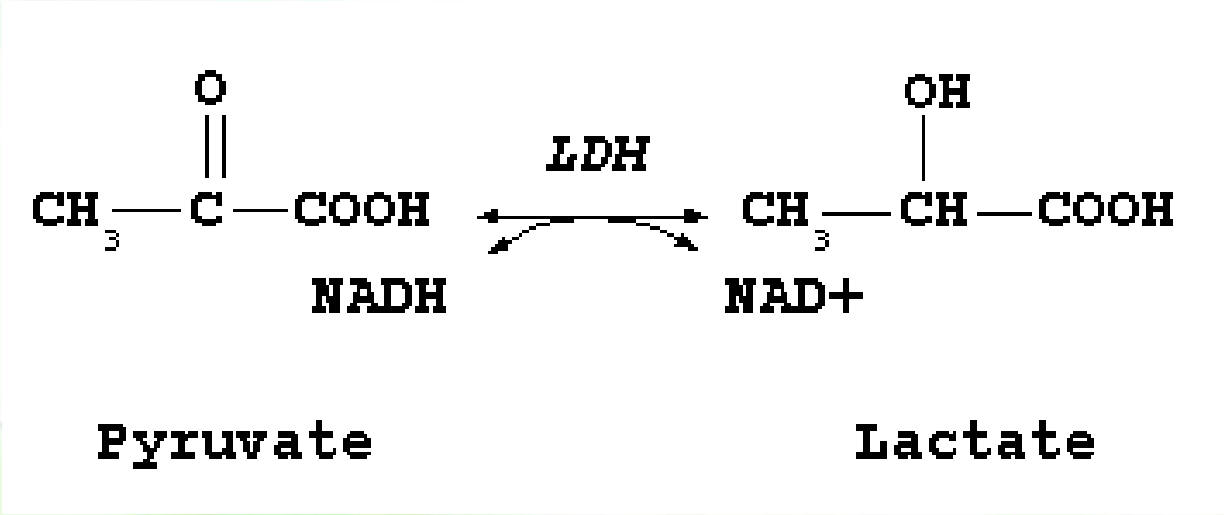
Logic behind tissue distribution



Isoenzyme	Structure	Present in	Elevated in
LDH1	(H_4)	Myocardium	myocardial infarction
LDH2	(H_3M_1)	RBC	
LDH3	(H_2M_2)	Lungs	
LDH4	(H_1M_3)	Kidney	
LDH5	(M_4)	Skeletal muscle, Liver	Skeletal muscle and liver diseases

Function of isozymes

- Muscles can function anaerobically, but heart tissues cannot.
- Whereas the all-M isozyme (M4) functions anaerobically and catalyzes the reduction of pyruvate into lactate, the all-H enzyme (H4) functions aerobically and catalyzes the reverse reaction.



Regulation of LDH

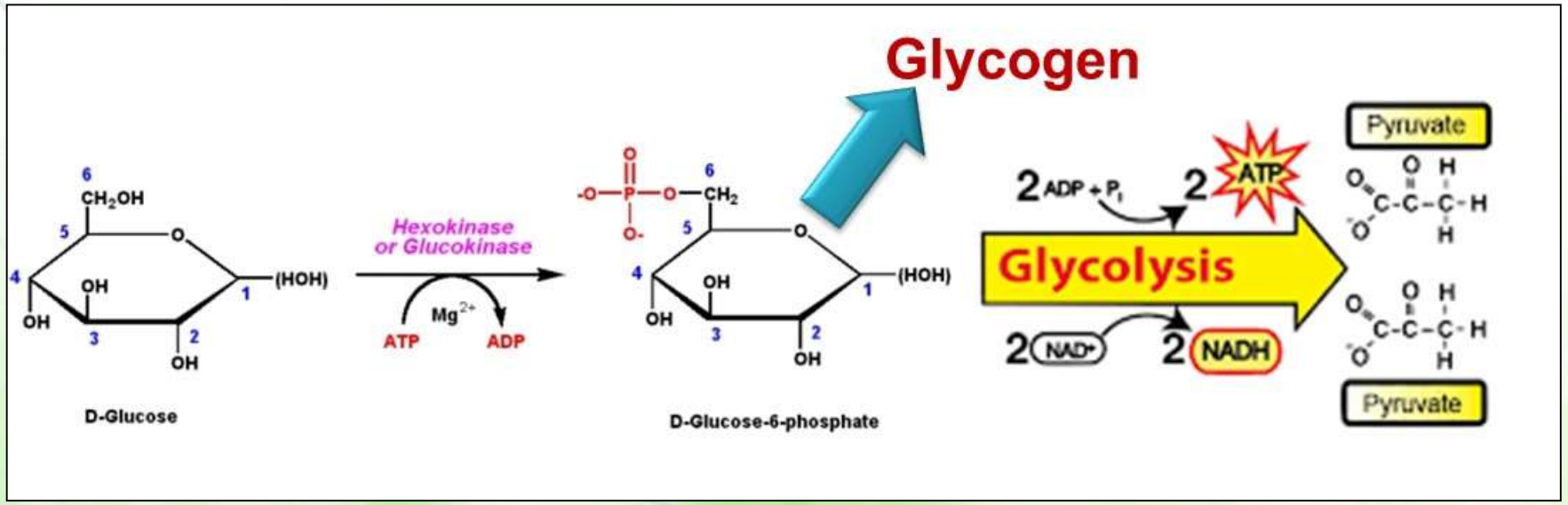
- H4 LDH has a low Km for lactate, high Km for pyruvate, and is inhibited by high levels of pyruvate.
  - The H4 isoenzyme favors (lactate to pyruvate).
- The M4 LDH enzyme has a high Km for pyruvate and is not inhibited by pyruvate.
  - M4 LDH is always active even at high levels of pyruvate ensuring that pyruvate is always funneled to anaerobic metabolism.

Hexokinase vs glucokinase

- Hexokinase and glucokinase (hexokinase IV) are allosteric isozymes that catalyze:

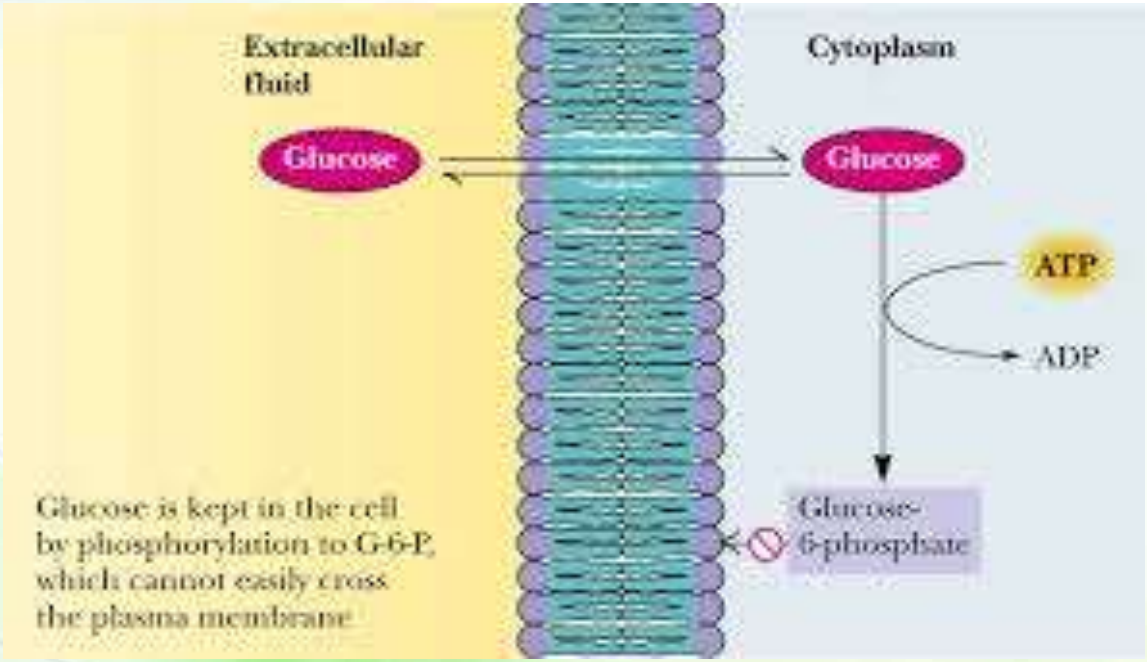
  Glucose → Glucose-6-Phosphate

- Glucokinase is a liver (and pancreatic) enzyme, whereas hexokinase is هى RBC (and skeletal muscle) enzyme.
  - The purpose of liver glucose is to balance glucose level in the blood.
  - The purpose of RBC glucose is to produce energy.



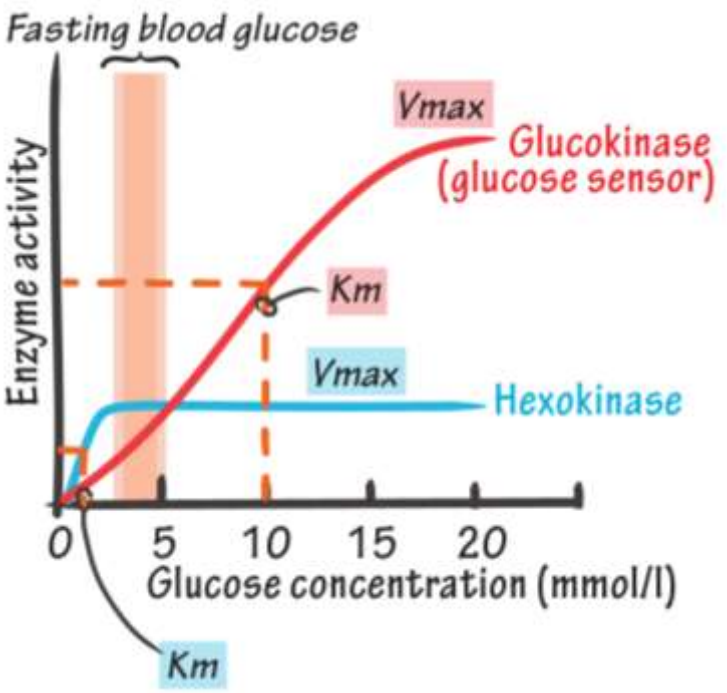
Biological significance

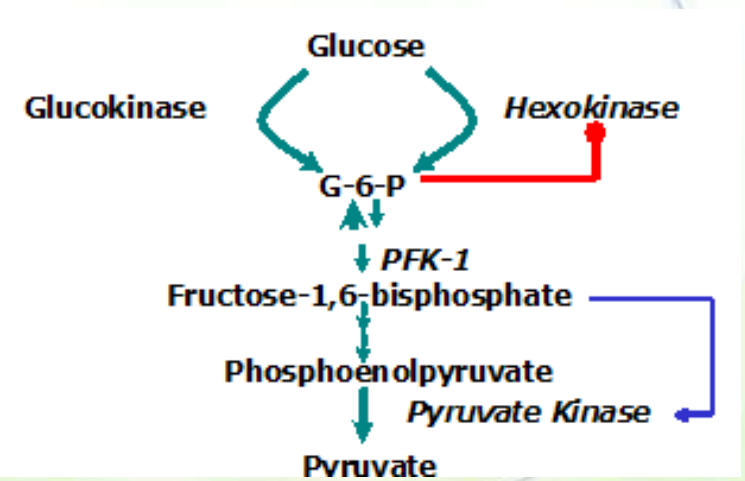
- Note: once glucose is phosphorylated, it cannot cross plasma membrane out of cells.
  - Liver: low efficiency enzyme to provide glucose to other organs.
  - RBC and skeletal muscles: high efficiency enzyme to trap glucose.



Regulation of hexokinase and glucokinase

- Note Vmax and K_M values (low - 0.1 mM for hexokinase and (high - 10 mM for glucokinase)
- Regulation
  - Hexokinase is inhibited by glucose-6-phosphate, but glucokinase is not.
  - Glucokinase is activated by insulin and inhibited by glucagon.
- Significance:
  - At fasting state, glucose is not stored.
  - At well-fed state, RBCs and skeletal muscles do not consume all glucose in blood and liver can convert excess glucose in glycogen for storage.





Regulation of enzymatic activity

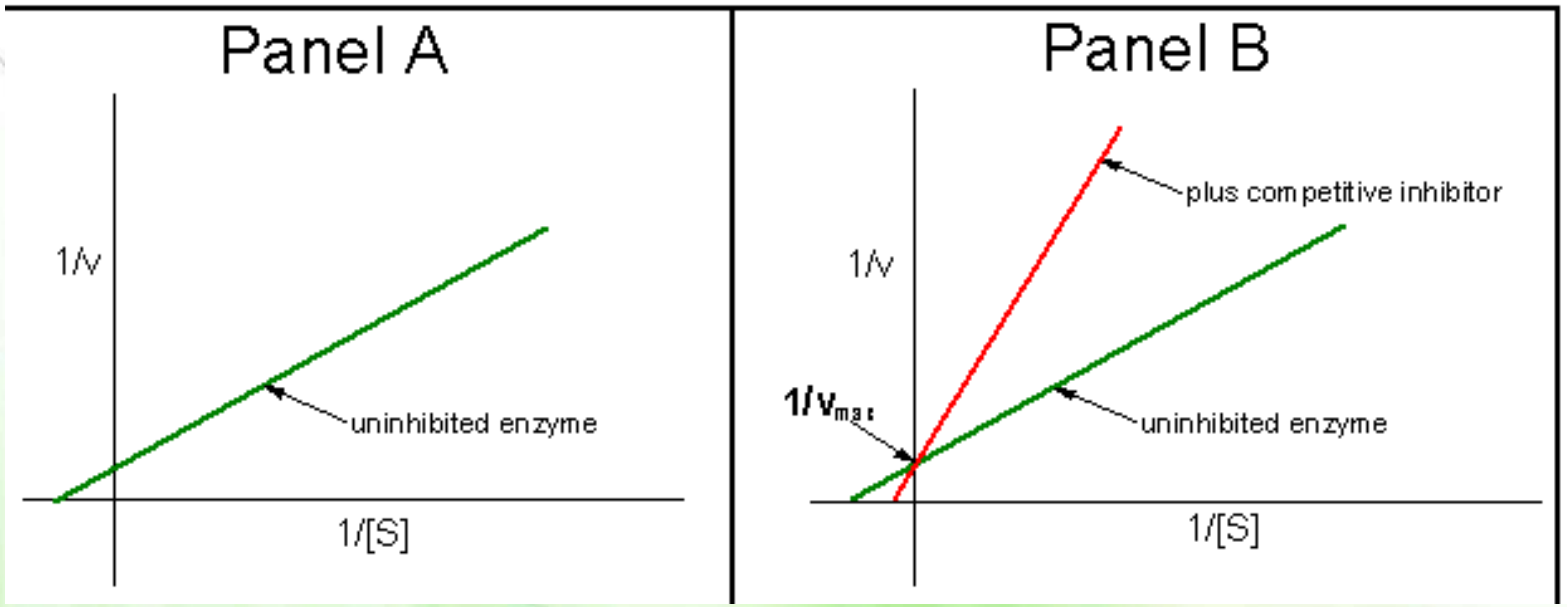
Inhibitors

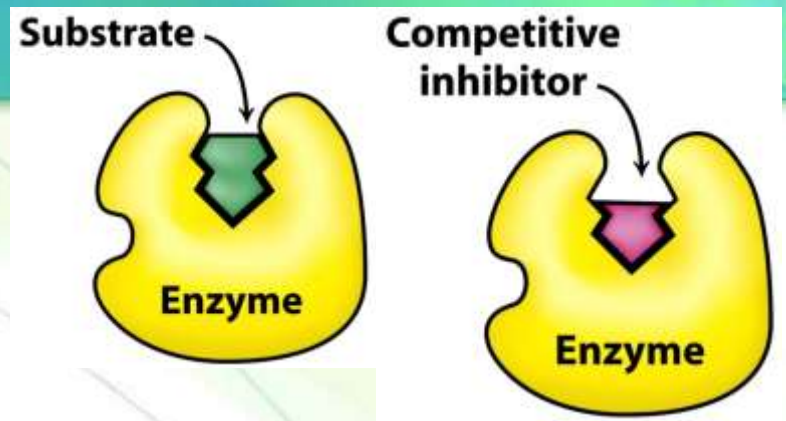
Enzyme inhibitors

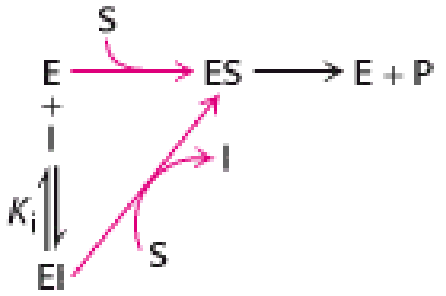
- Enzyme inhibition can be either reversible or irreversible.
  - Reversible inhibitors rapidly dissociate from enzymes (e.g. non-covalent binding).
    - Competitive, noncompetitive, or uncompetitive inhibition.
  - An irreversible inhibitor is tightly bound (e.g. covalently) to the enzyme.
    - Lower concentration of active enzyme.

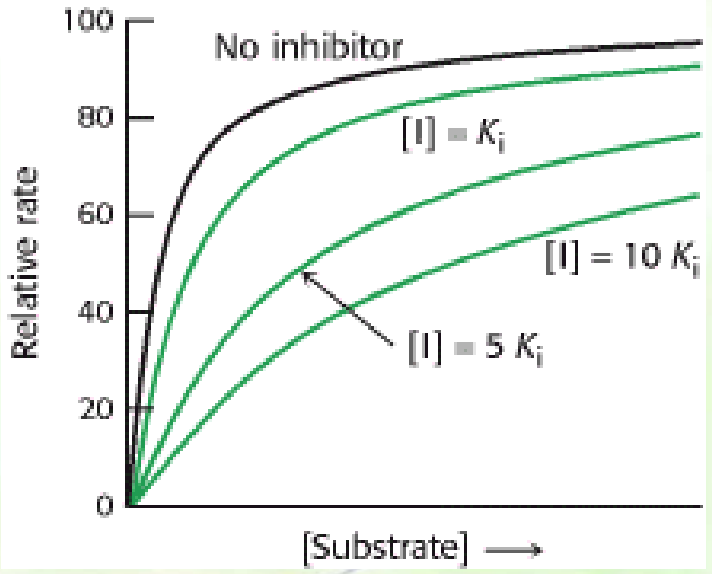
Competitive inhibition

- Competitive inhibitors compete with the substrate for the active site.
  - Increasing substrate can overcome inhibition.
- Same Vmax, but higher K_M



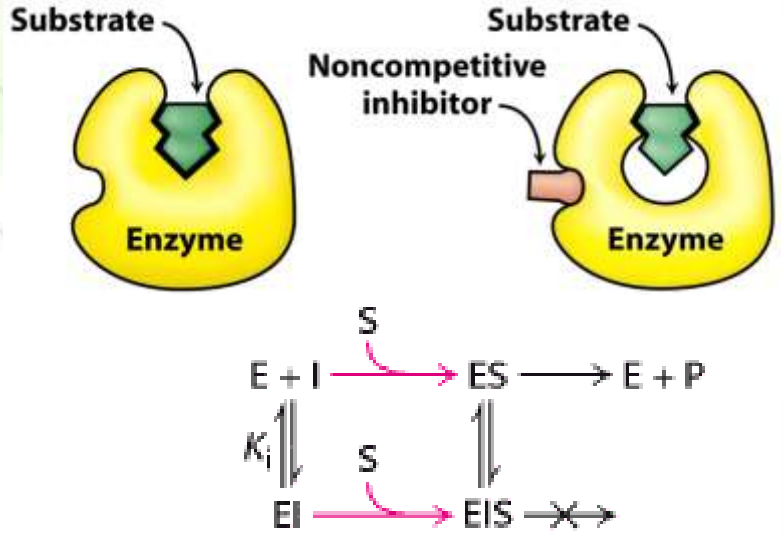


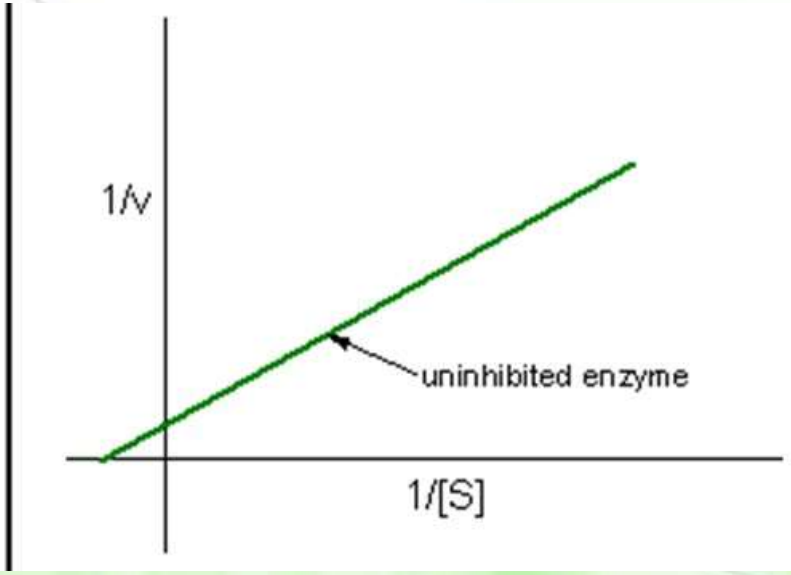


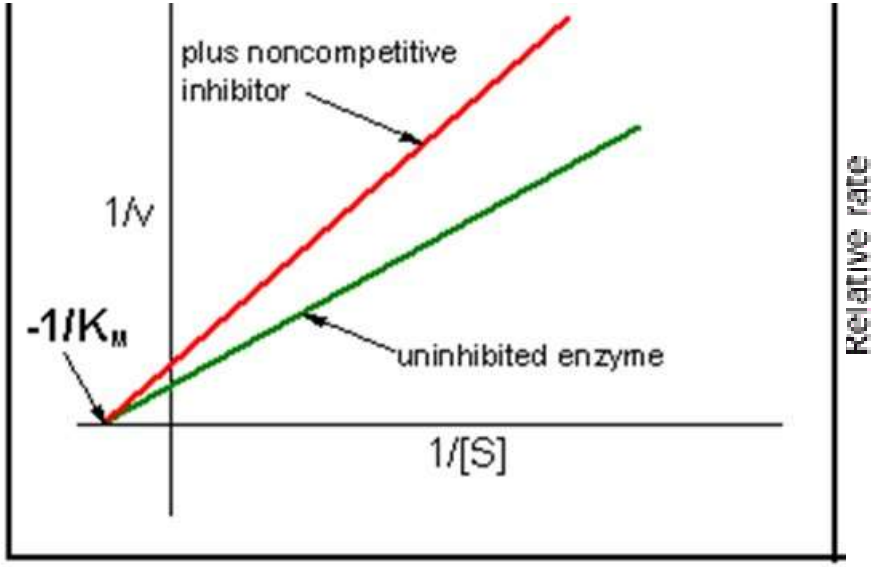


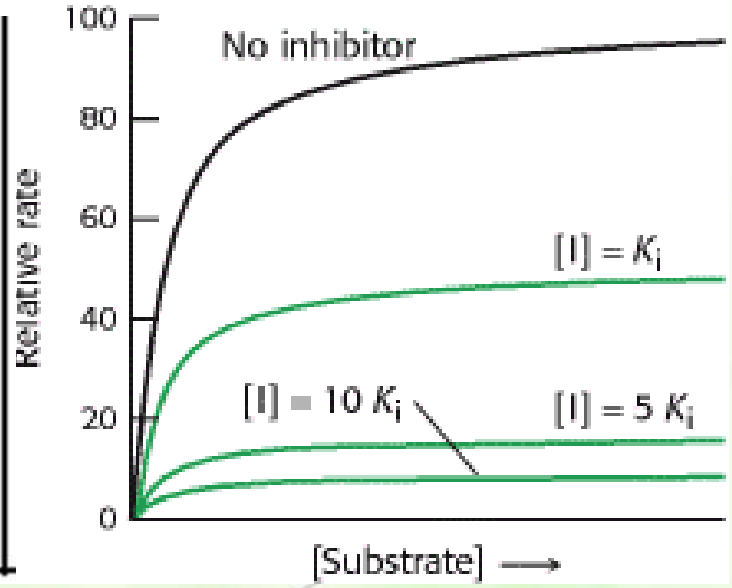
Noncompetitive inhibition

- Noncompetitive inhibitors bind E or ES complex at a site other than the catalytic site.
- Substrate can bind to the enzyme-inhibitor complex, but ESI cannot form a product.
- Lower Vmax, but same K_M

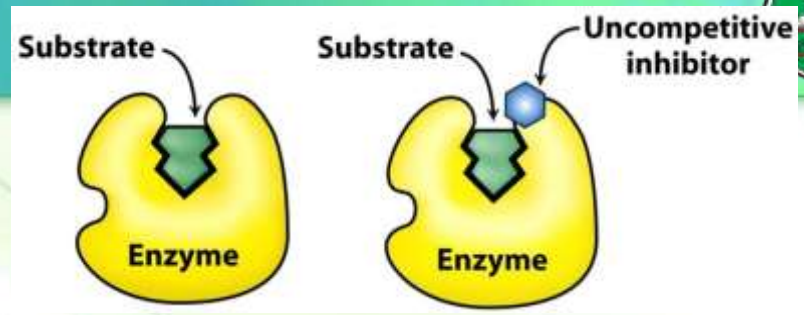






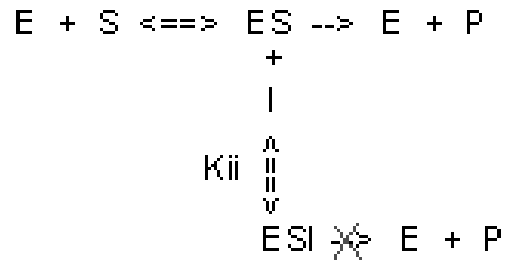


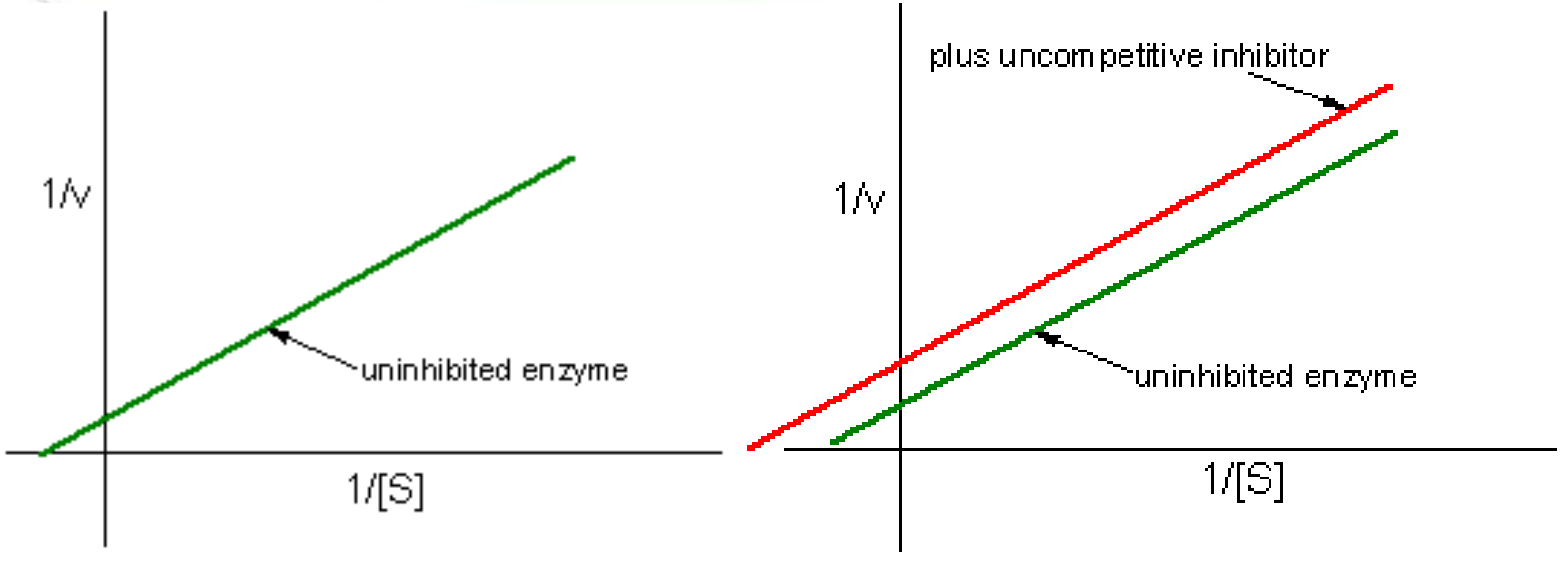
Uncompetitive inhibition

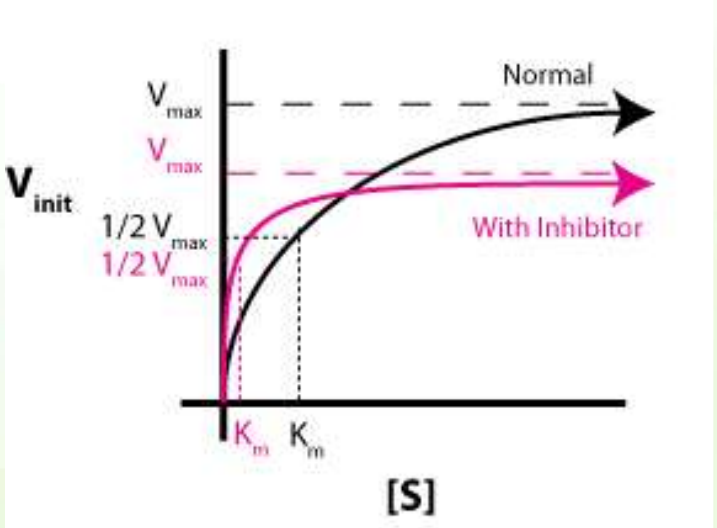


UNCOMPETITIVE

- Uncompetitive inhibitors bind to the enzyme-substrate complex only reducing both Vmax and K_M.







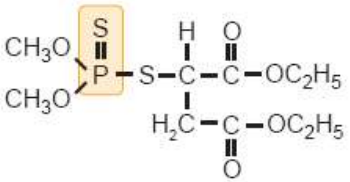
Mechanism-based inhibitors

Irreversible inhibitors

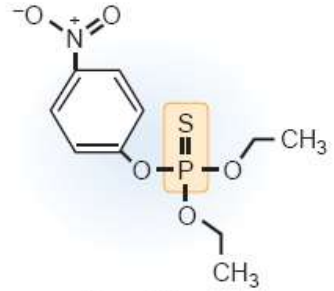
- Mechanism-based inhibitors mimic or participate in an intermediate step of the catalytic reaction.
- They include:
  - Covalent inhibitors
  - Transition state analogs
  - Heavy metals
- Irreversible inhibitors decrease the concentration of active enzyme.

Covalent inhibitors

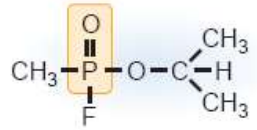
- They form covalent or extremely tight bonds with active site amino acids.
  - Example: diisopropyl fluorophosphate (DFP) is a organophosphate
    - The nerve gas sarin
    - The insecticides malathion & parathion.
    - DFP inhibits acetylcholinesterase preventing the degradation of the neurotransmitter acetylcholine.



Malathion

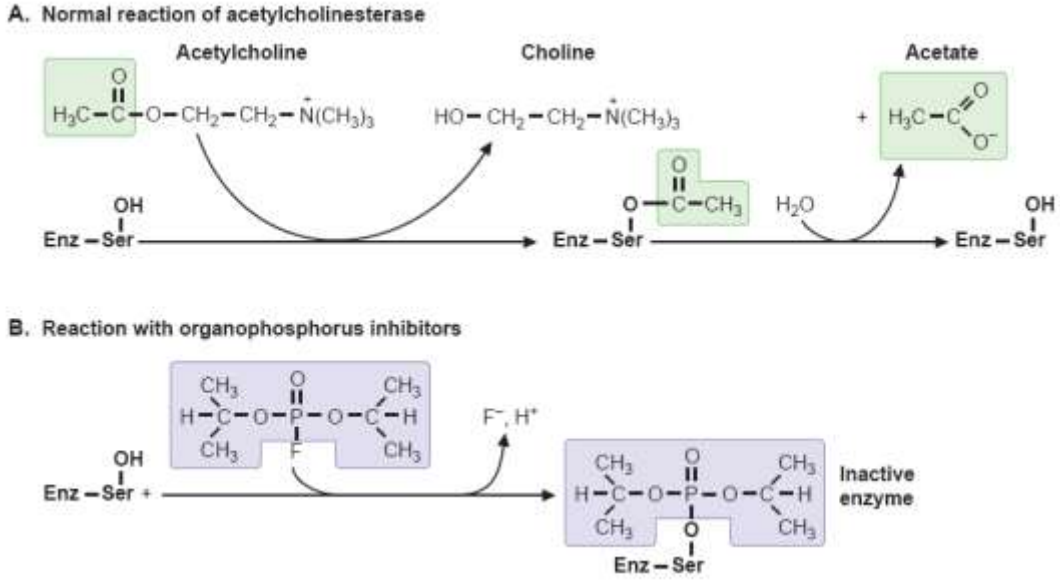


Parathion



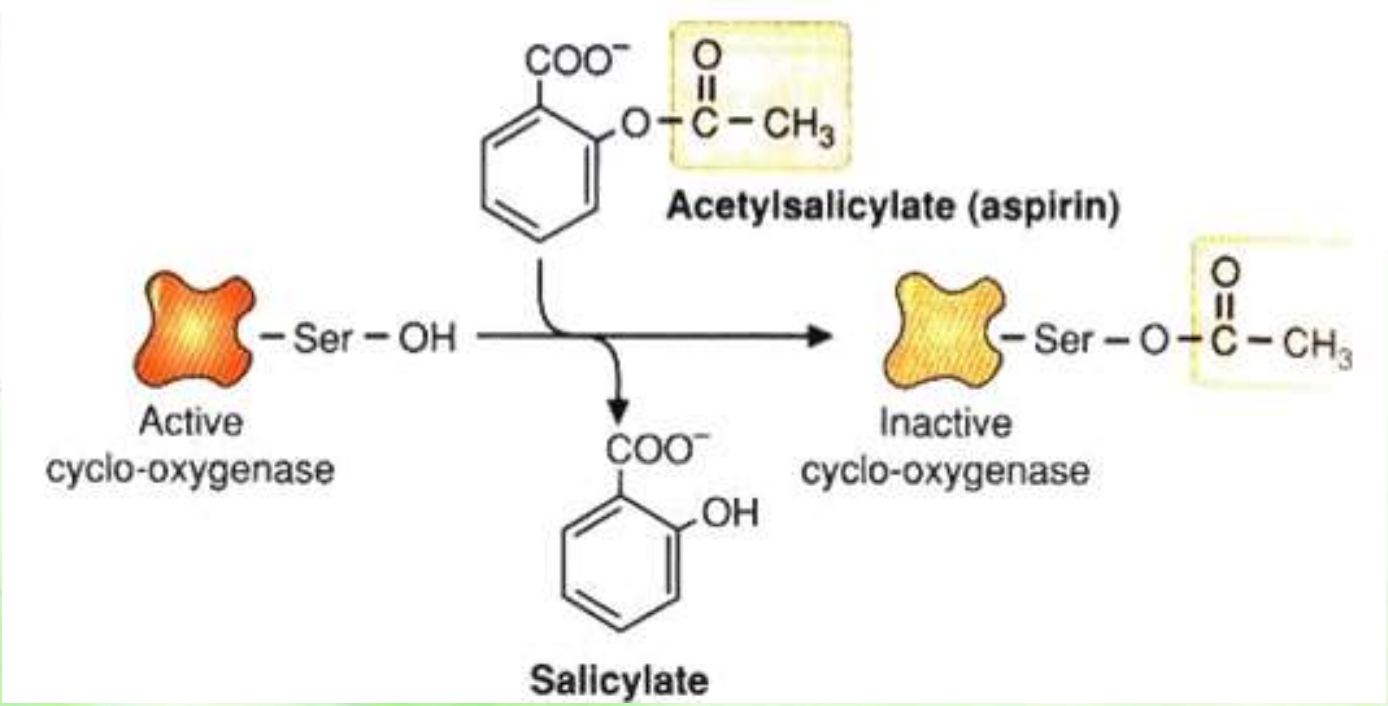
Sarin

DFP also inhibits other enzymes that use serine (ex. serine proteases), but not lethal.



Aspirin

- Aspirin (acetylsalicylic acid) acetylates an active site serine of cyclooxygenase.
- Aspirin resembles a portion of the prostaglandin precursor that is a physiologic substrate for the enzyme.



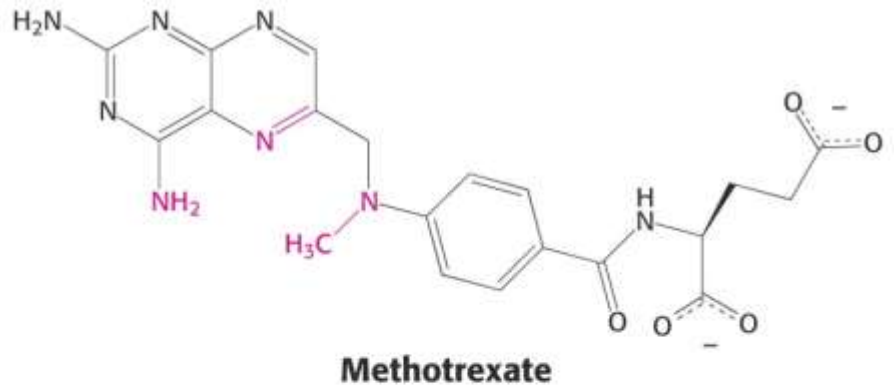
Substrate and transition-State analogs

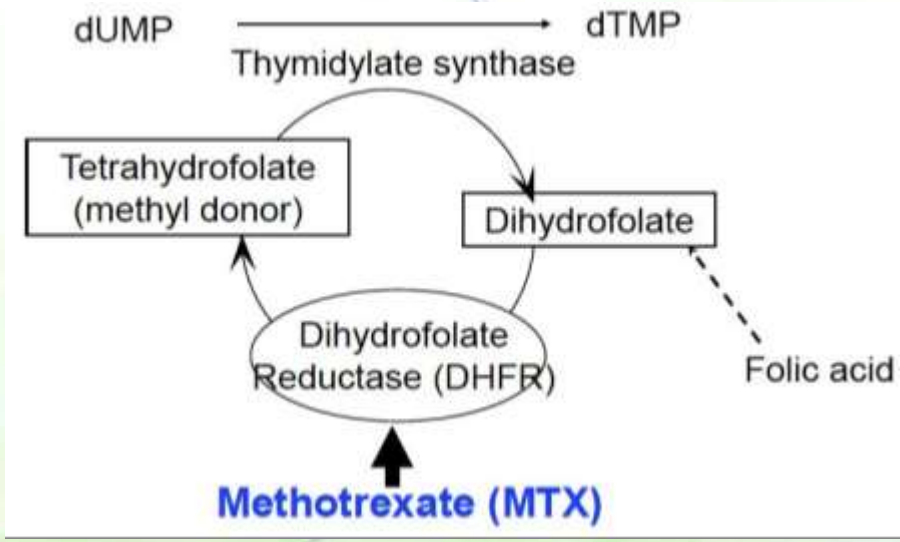
Suicide inhibitors

- They bind more tightly than substrates.
- Drugs cannot be designed that precisely mimic the transition state! (highly unstable structure).

Methotrexate

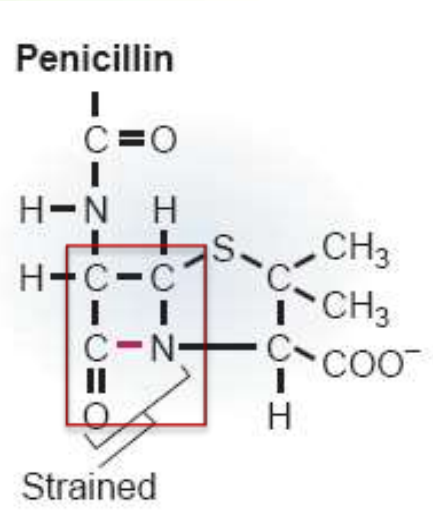
- Methotrexate is a synthetic inhibitor used to treat cancer.
- It is a structural analog of folate, a substrate for the enzyme dihydrofolate reductase, and a coenzyme for thymidylate kinase , both of which are responsible for the synthesis of nucleotides.
- It binds to dihydrofolate reductase 1000-fold more tightly than the natural substrate and inhibits nucleotide base synthesis.

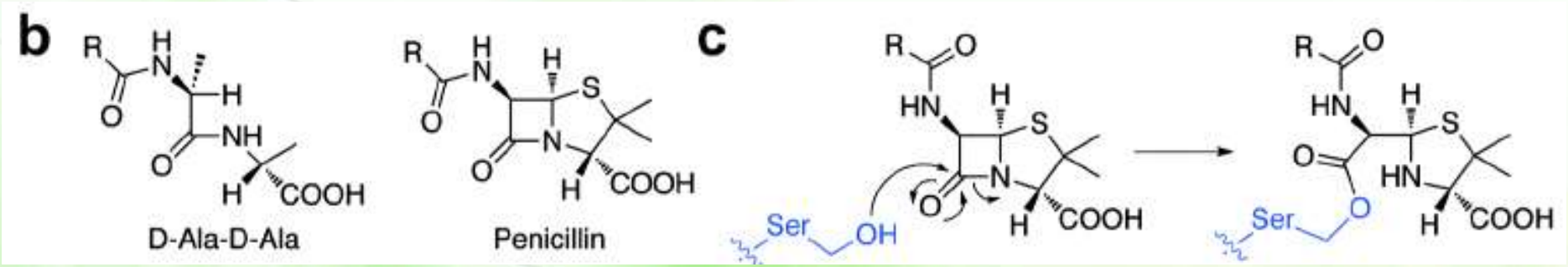




Penicillin

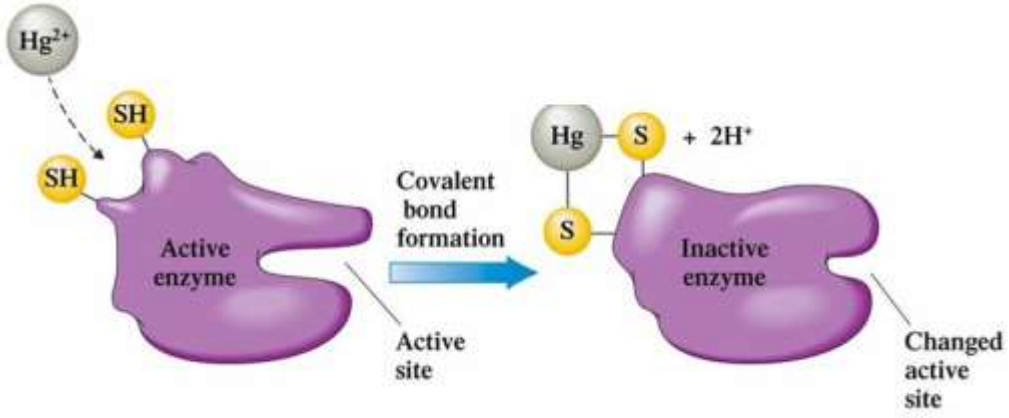
- The cell wall is the outer covering of the bacteria-containing peptidoglycan layer which is made up of peptides that are cross-linked by glycopeptidyl transpeptidase.
- The amide bond in the β-lactam ring of penicillin looks like the natural transition-state complex.
  - Penicillin is an irreversible, transition-state analog inhibitor of the enzyme.
- The active site serine attacks the highly strained β -lactam ring.





Heavy Metals

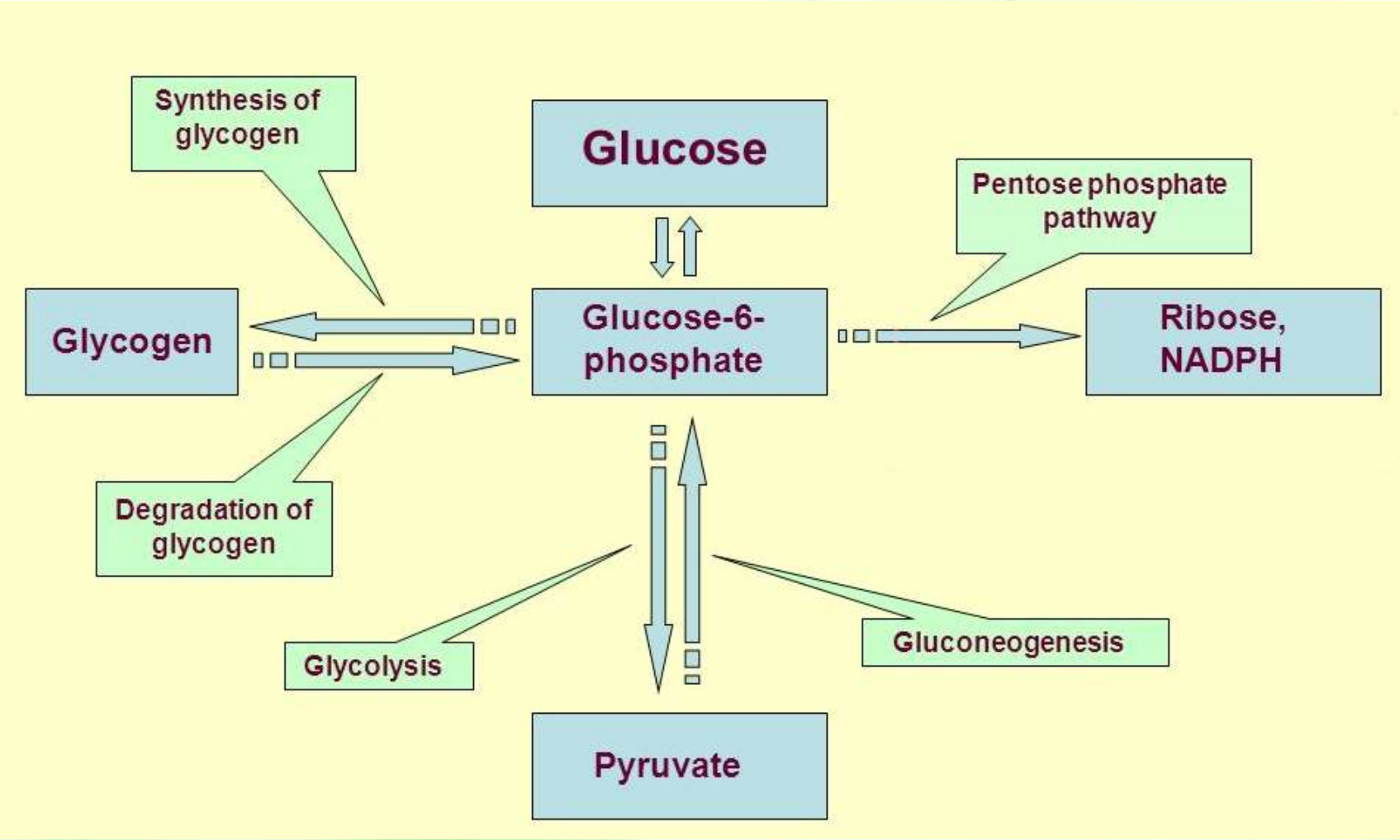
- Mercury (Hg), lead (Pb), aluminum (Al), or iron (Fe) result in tight binding to a functional group in an enzyme .
  - Nonspecific inhibition at high doses.
- Mercury binds to reactive sulfhydryl groups away from the active site and affect the binding of substrates.
  - Unknown enzymes are inhibited in mercury toxicity.



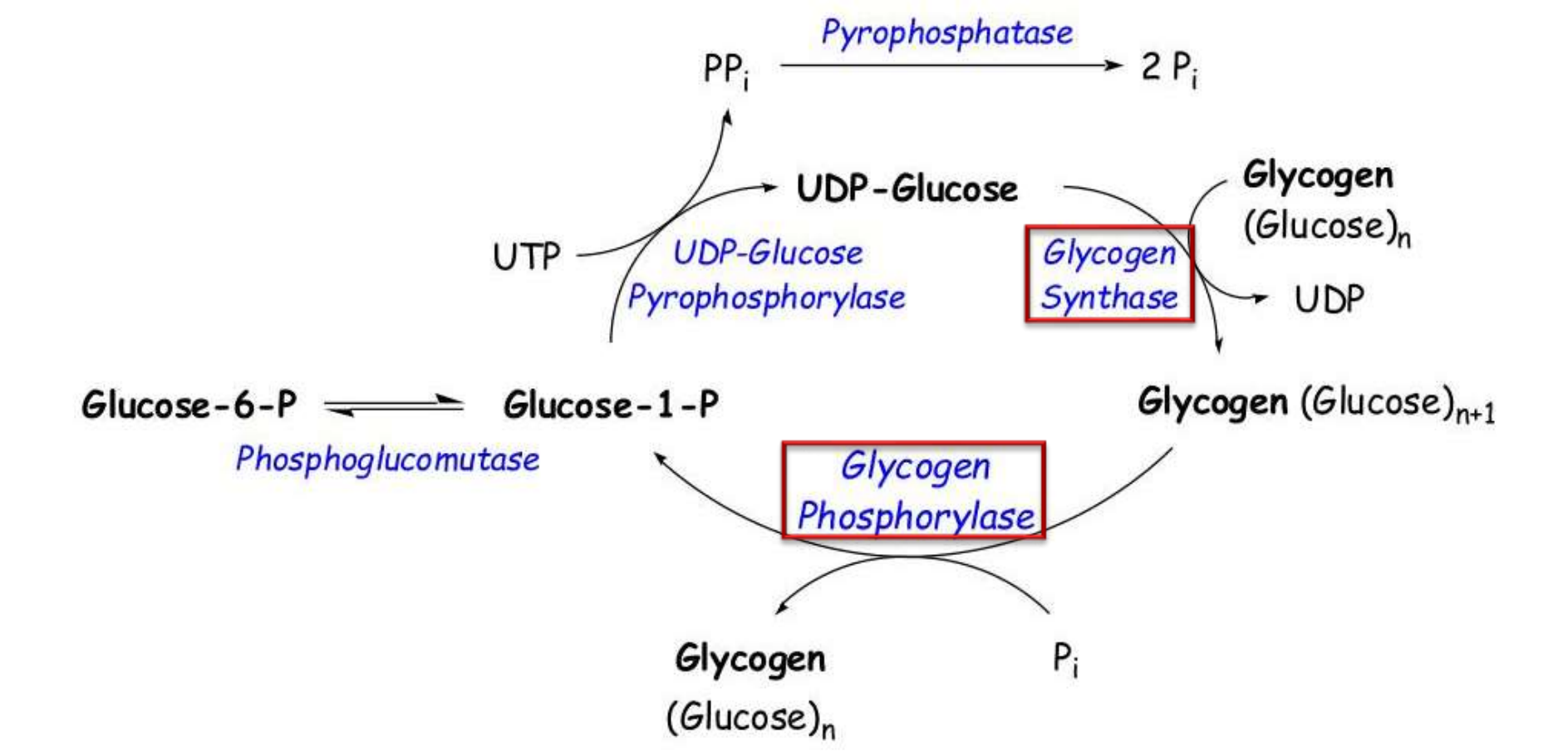
- Lead replaces the normal functional metal in an enzyme such as calcium, iron, or zinc by an irreversible mechanism.
  - Its developmental & neurologic toxicity may be caused by its ability to replace Ca+2 in several regulatory proteins that are important in the central nervous system and other tissues.

Regulation through conformational changes

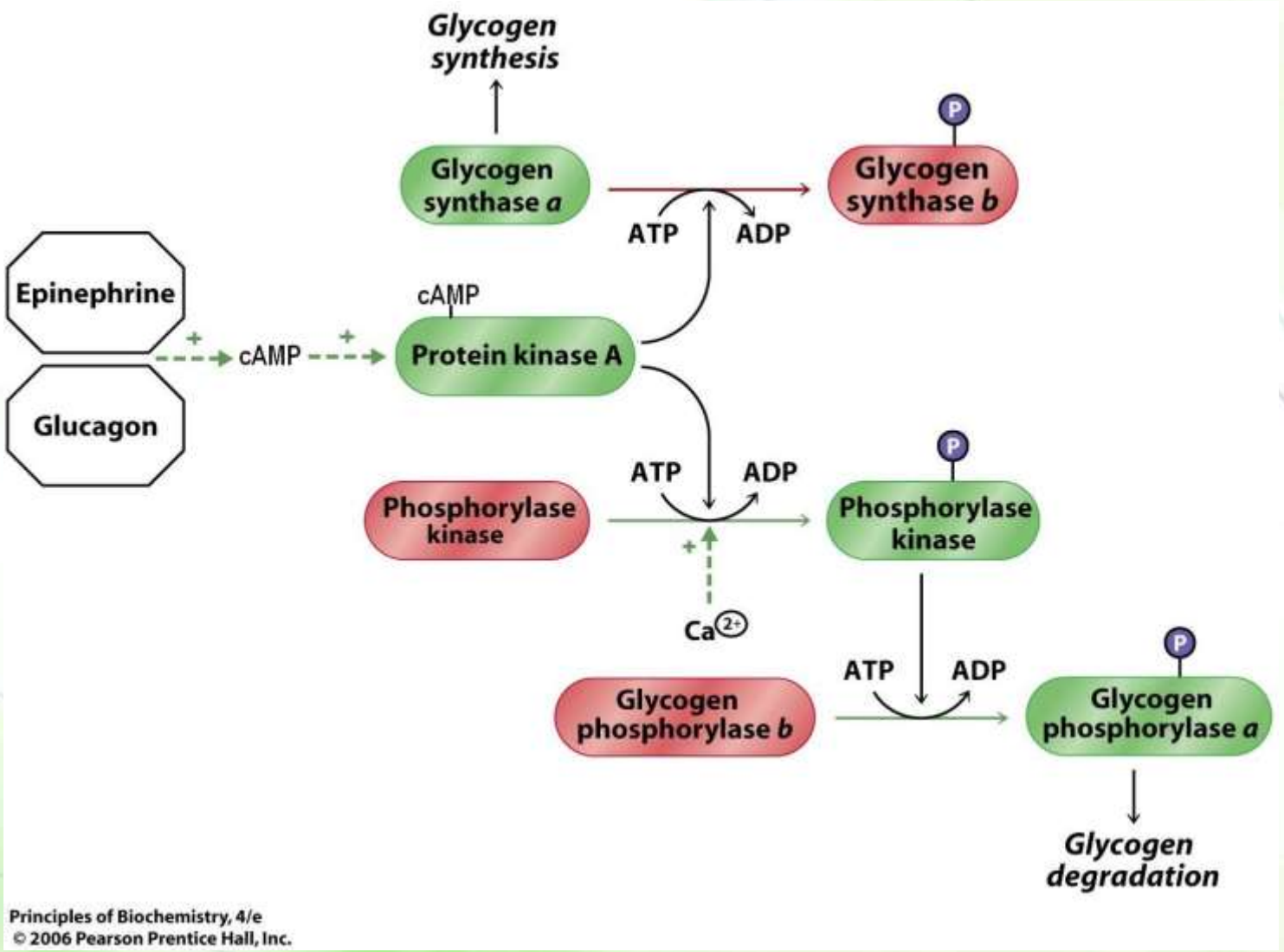
The different fates of glucose



Metabolism of glycogen

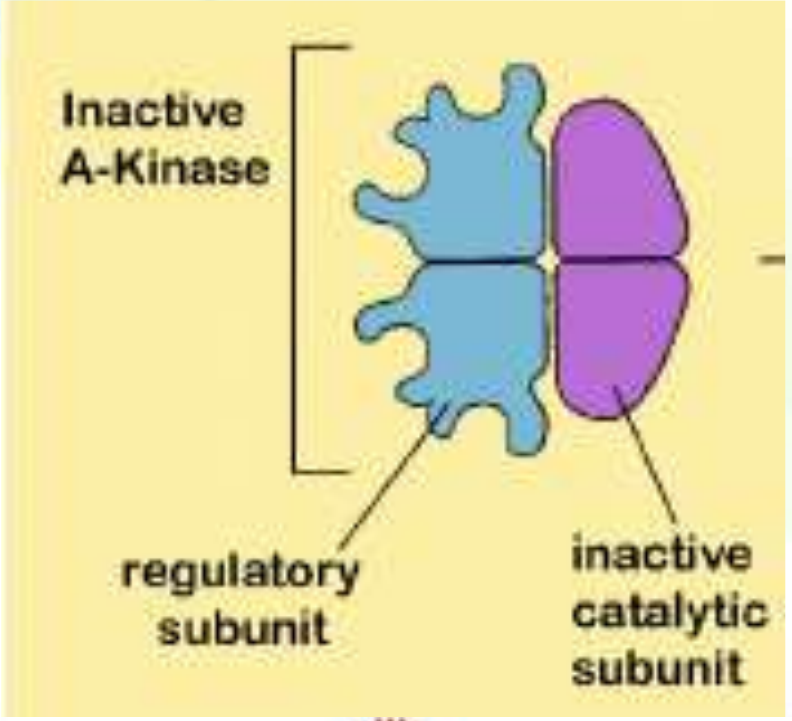


Regulation by phosphorylation

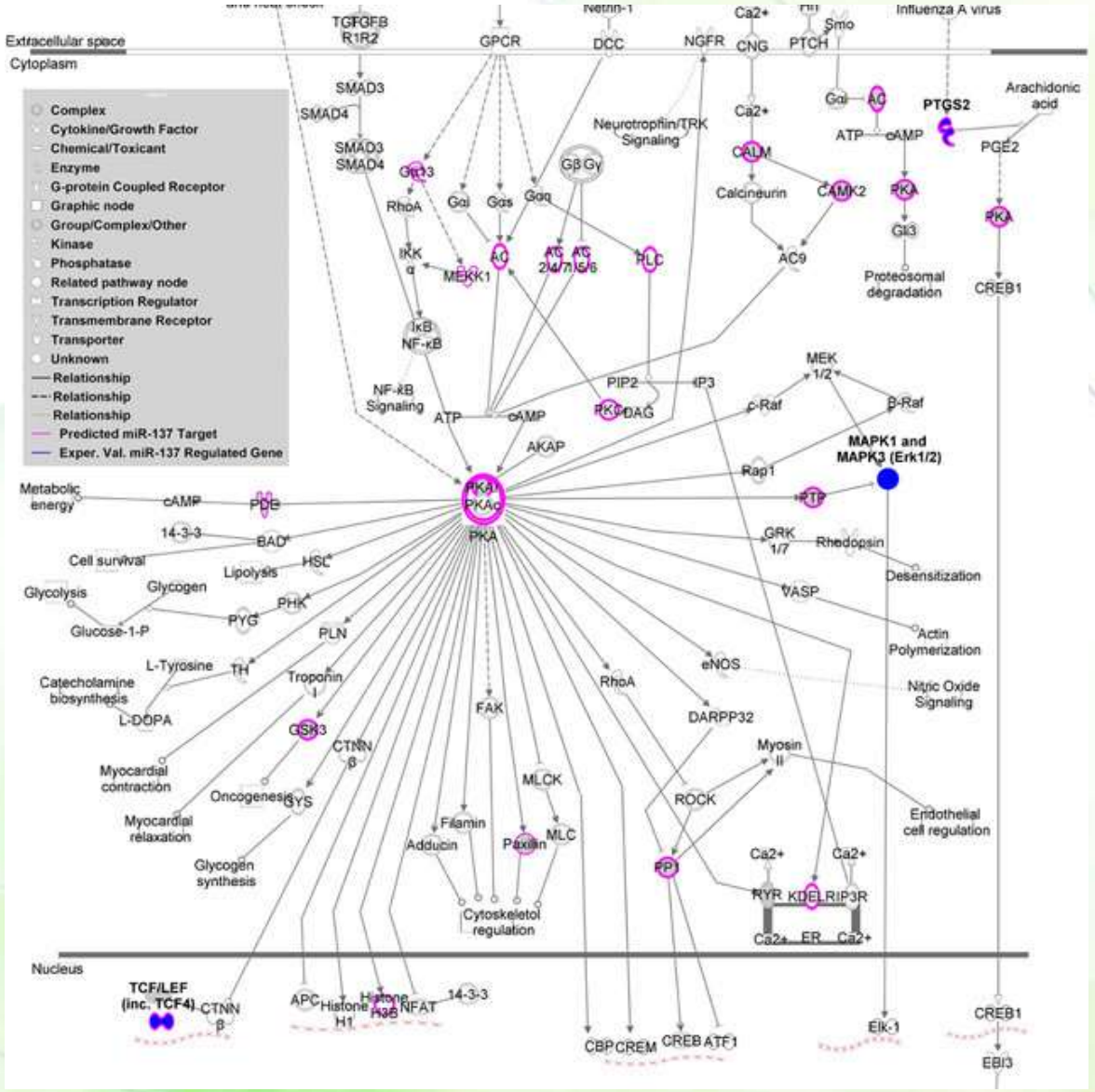


PKA-structure and regulation

- When inactive, protein kinase A (PKA), a serine/threonine kinase, consists of four subunits (R2C2).
  - Two regulatory (R) subunits with high affinity for cAMP,
  - Two catalytic (C) subunits
- PKA phosphorylates several enzymes that regulate different metabolic pathways.
  - Example: glycogen phosphorylase kinase

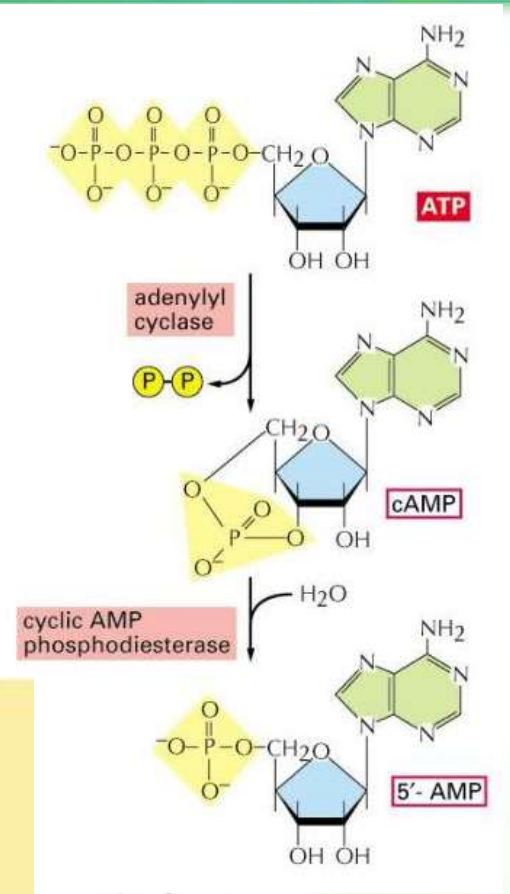


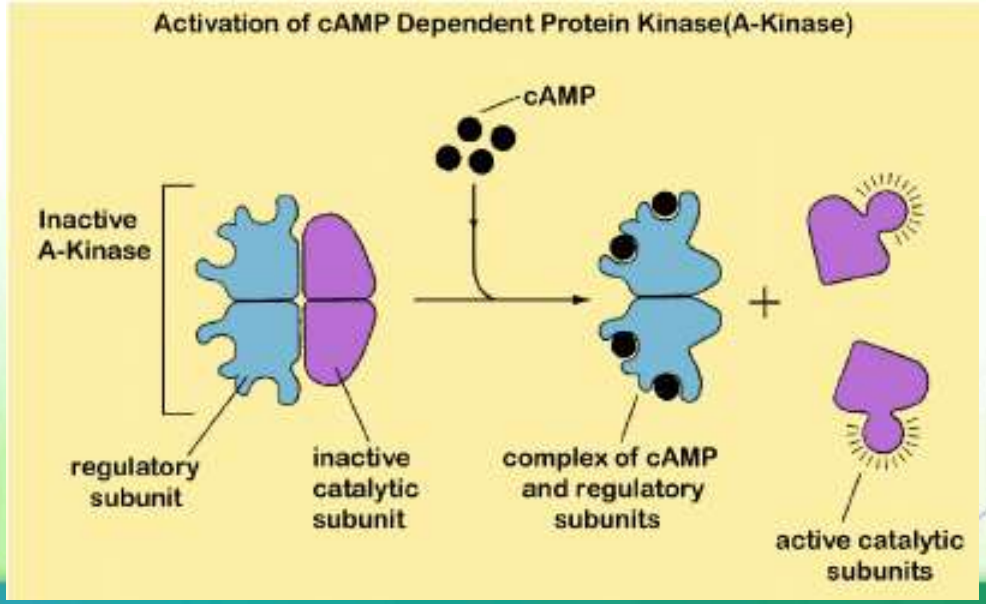
The many targets of PKA



cAMP and protein kinase A (PKA)

- Small-molecule modulators can have dramatic effects on enzymes.
- For example, cAMP, which is structurally modified AMP, can activate protein kinase A (PKA).
- The binding of two molecules of cAMP to each regulatory subunit leads to the dissociation of R2C2 into an R2 subunit and two active C subunits.

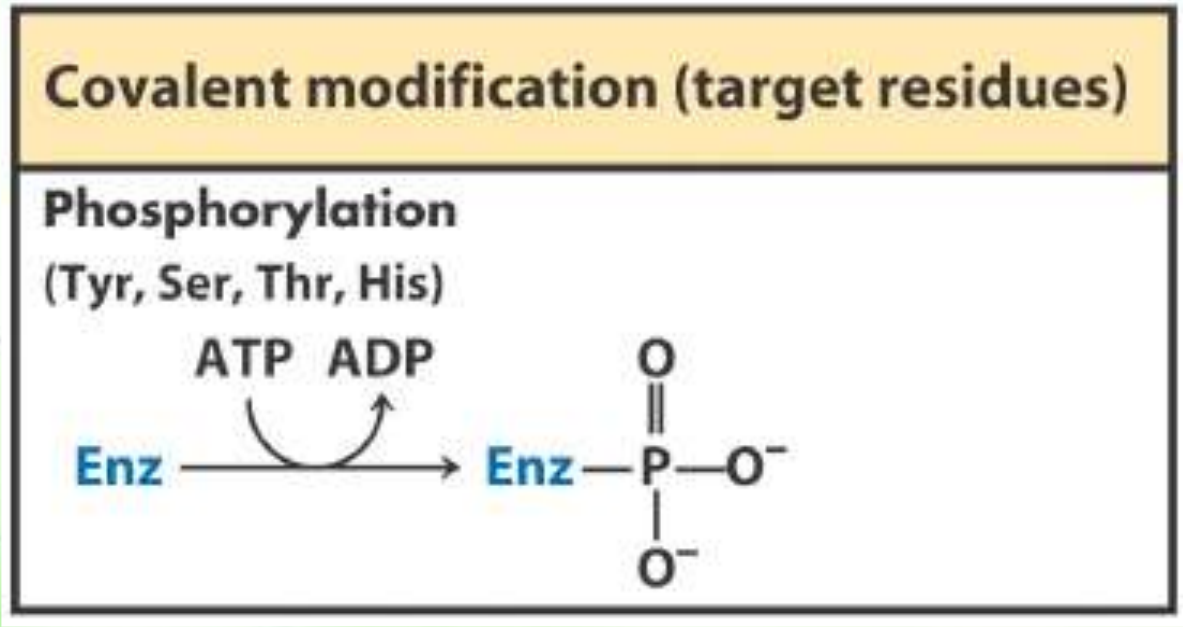




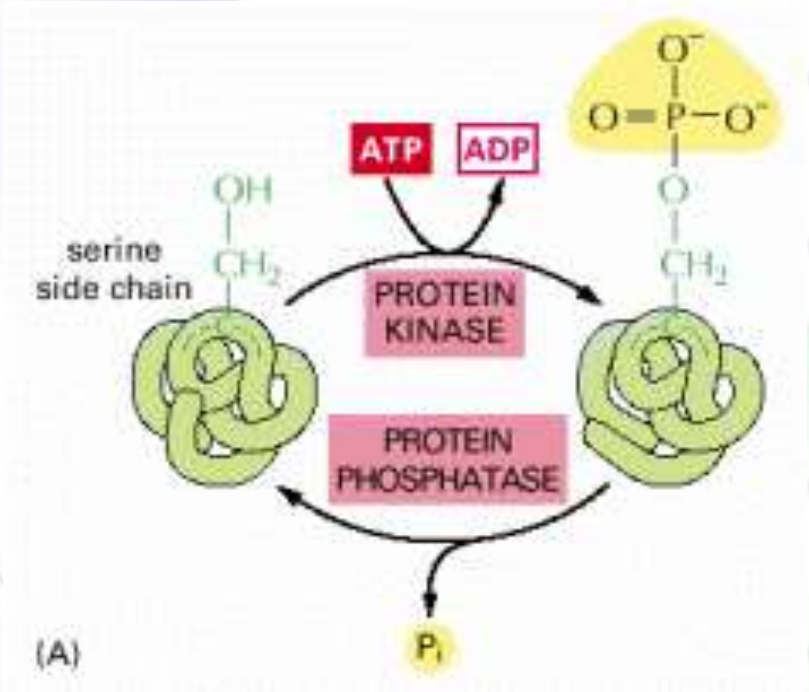
Reversible covalent modification

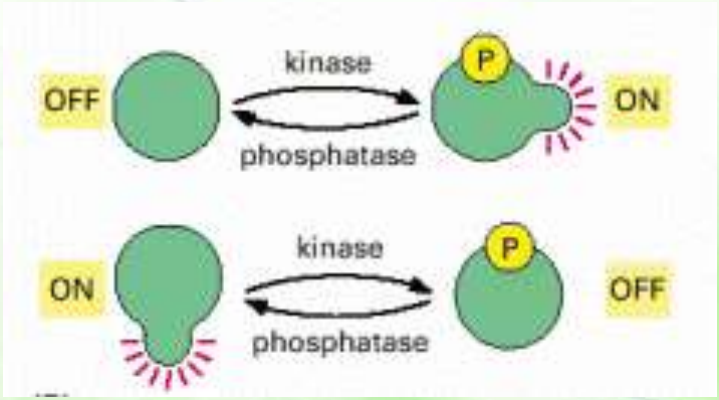
Advantage

- Rapid and transient
- A most common mechanism is enzyme phosphorylation (the covalent addition of a phosphate group to one of its amino acid side chains).
  - Usually serine, threonine, and tyrosine



Enzymes catalyzing (de)phosphorylation

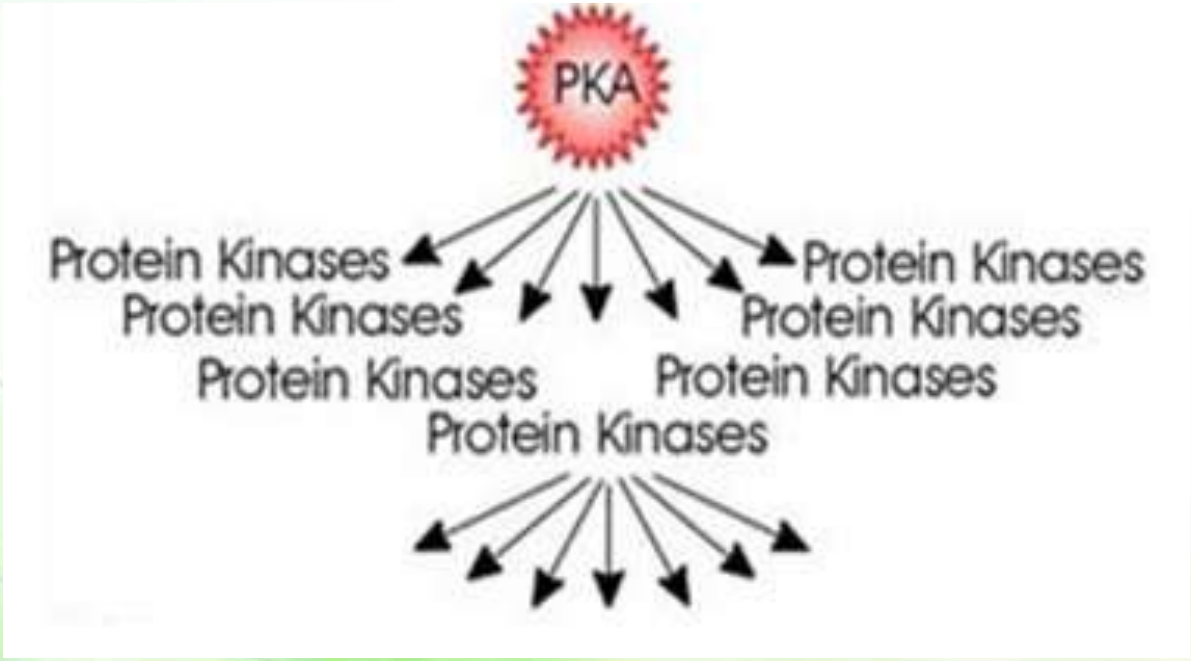




- ATP mostly is the phosphoryl donor in these reactions, which are catalyzed by protein kinases.
- The removal of phosphoryl groups (dephosphorylation) by hydrolysis is catalyzed by protein phosphatases.
- Note: dephosphorylation is not the reversal of phosphorylation.
- The addition or removal of a phosphate group to an enzyme may activate or inactivate these enzymes.

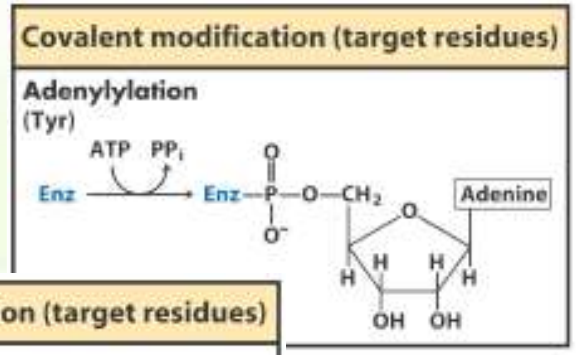
Why is phosphorylation effective?

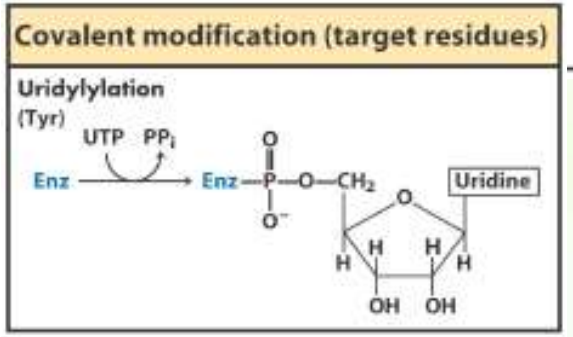
- Formation or removal of new electrostatic interactions and/or hydrogen bonds altering substrate binding and catalytic activity.
- It can happen in less than a second or over a span of hours.
- Phosphorylation often causes highly amplified effects.

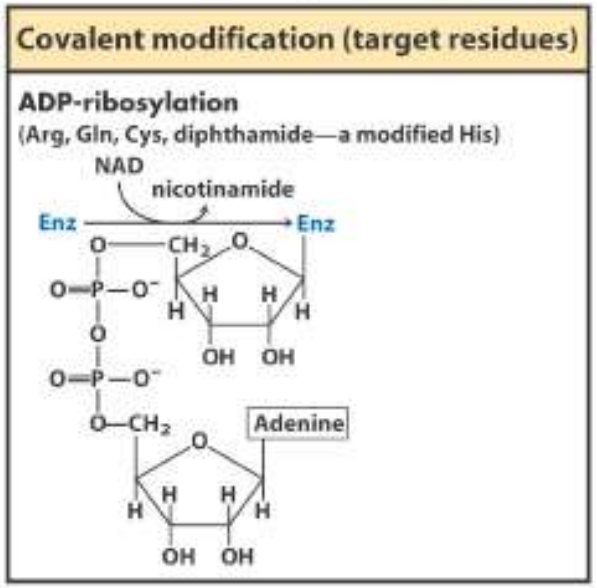


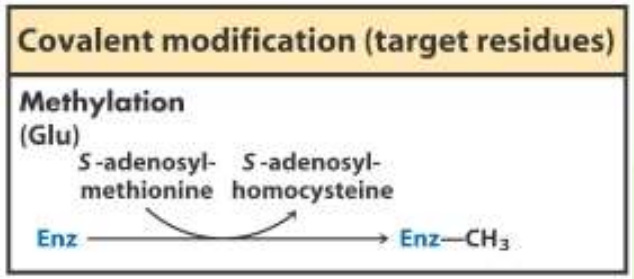
Others

- Adenylylation (addition of adenylyl group). AMP is transferred to Tyr residues through phosphodiester linkage.
- The addition of bulky AMP inhibits cytosolic enzymes.
- Uridylylation (addition of uridylyl group).
- ADP-ribosylation (addition of adenosine diphosphate ribosyl group) inactivates enzymes.
- Methylation of carboxylate side chains masking negative charges.
- Acetylation (from acetyl Co) to lysine residues masking positive charges.

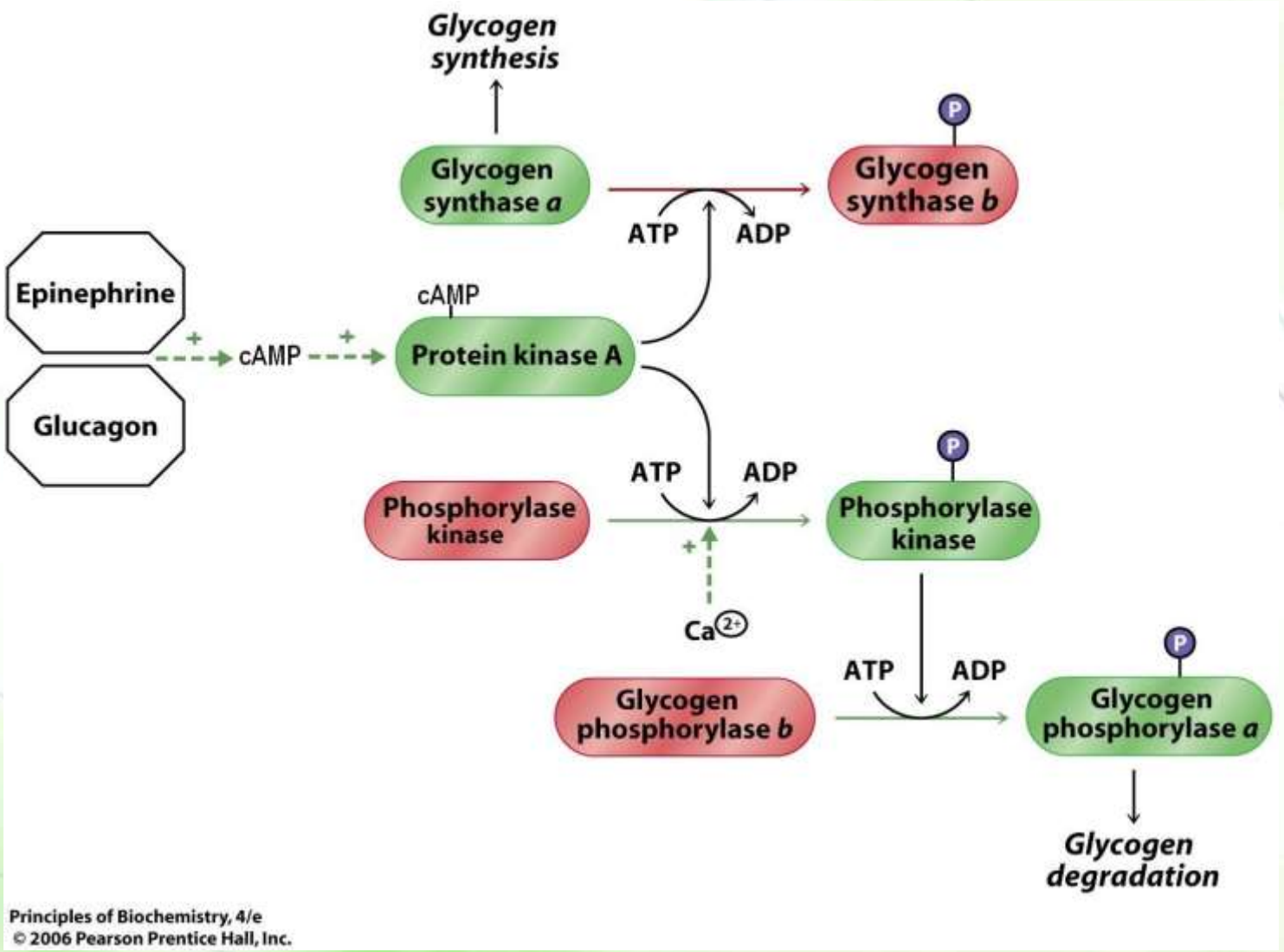








Phosphorylation cascade



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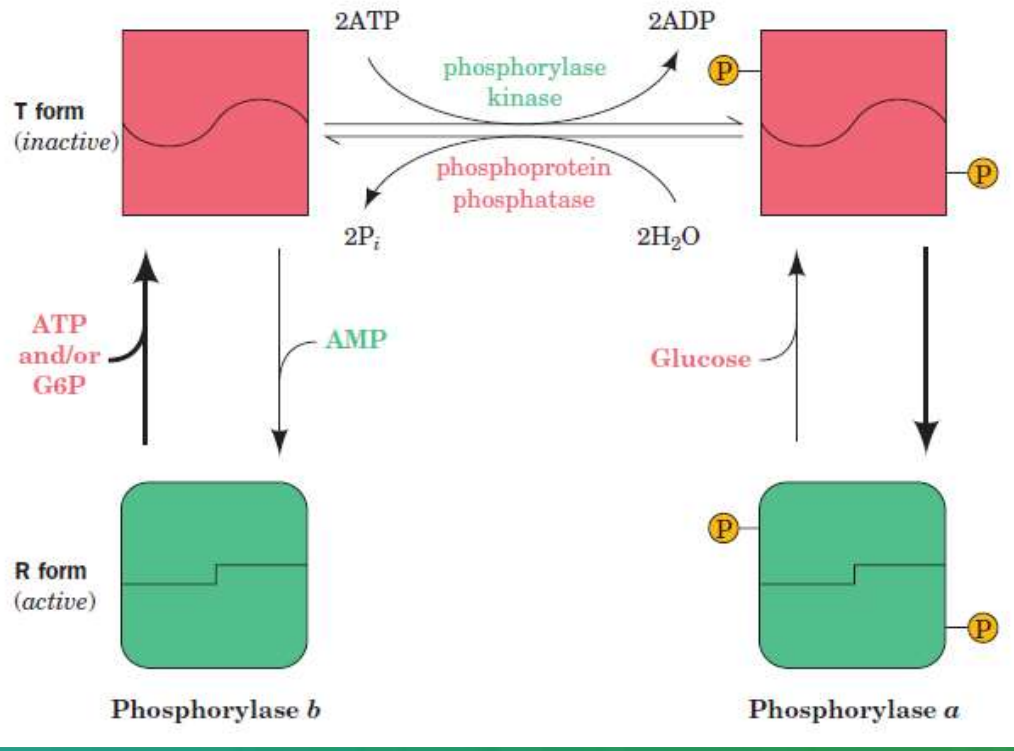
Example: Glycogen phosphorylase

$$\text{glycogen}_{(n\ \text{residues})} + P_i \longrightarrow \text{glycogen}_{(n-1\ \text{residues})} + \text{glucose 1-P}$$

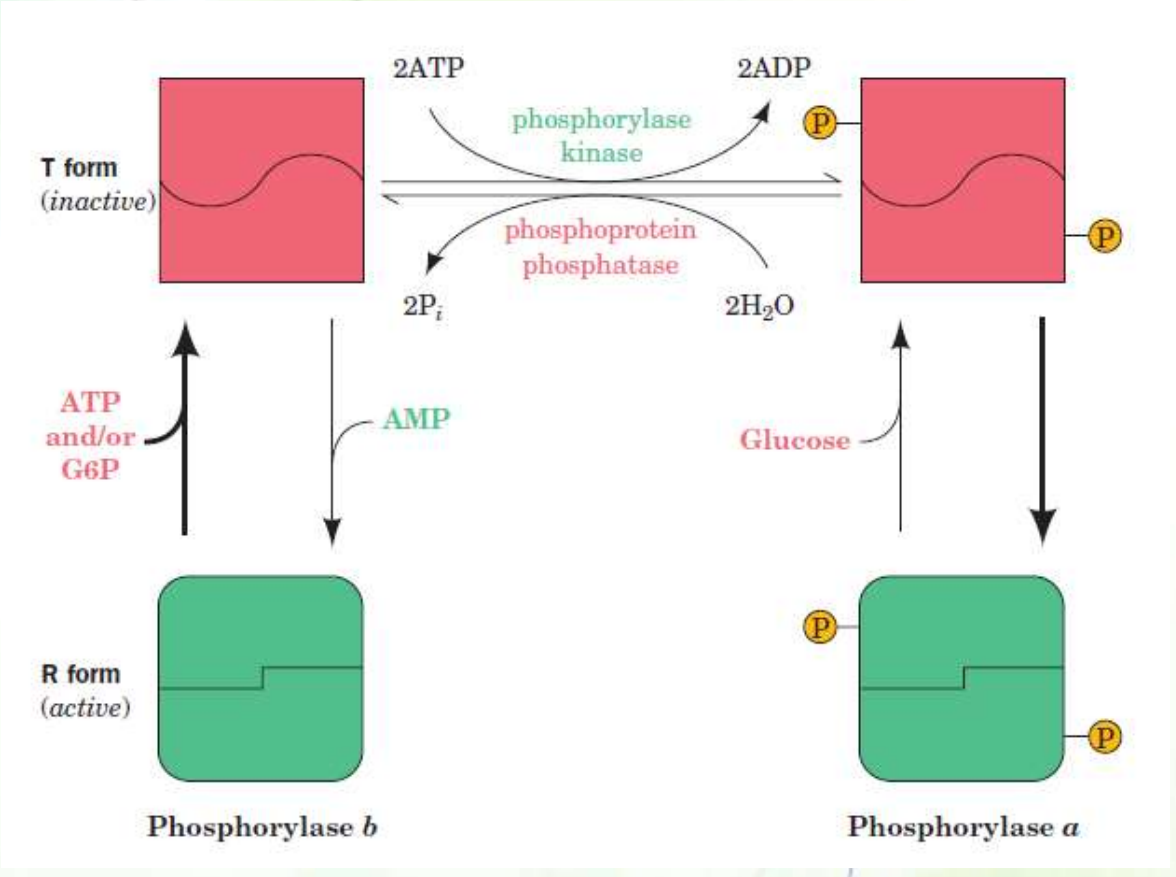
- GP catalyzes the removal of glucose molecules from glycogen.
- The phosphorylated Ser residue is remote from the active site.
- The enzyme exists in four forms:
  - T (inactive) or R (active) states and
  - Phosphorylated (a) or dephosphorylated (b)

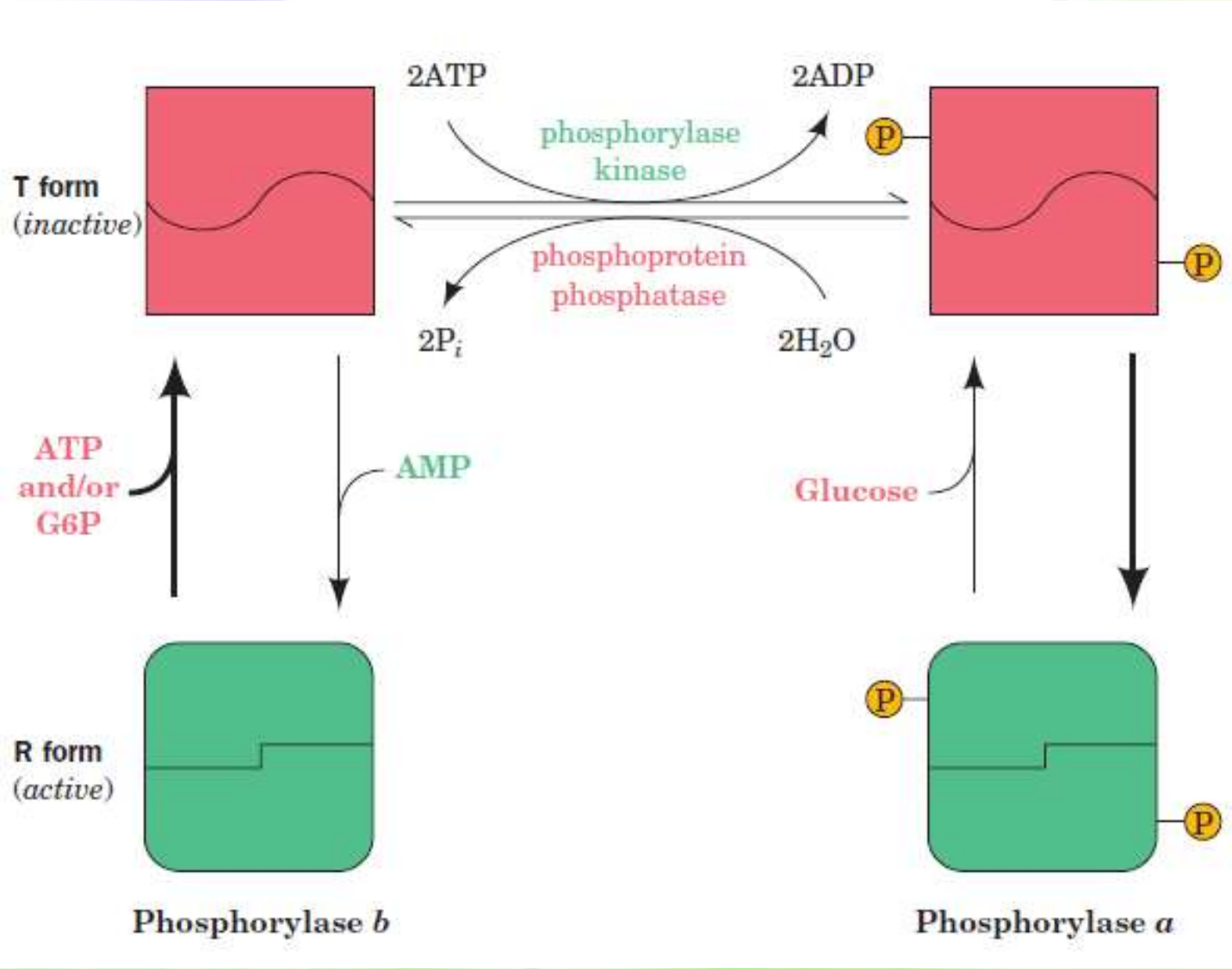
- *When phosphorylated, it is known as phosphorylase a.*
- *When dephosphorylated, it is known as phosphorylase b.*



The two forms of each form

- Both phosphorylase b and phosphorylase a exist in equilibrium between an active R state and a less-active T state.
- Phosphorylase b is usually inactive because the equilibrium favors the T state.
- Phosphorylase a is usually active because the equilibrium favors the R state.



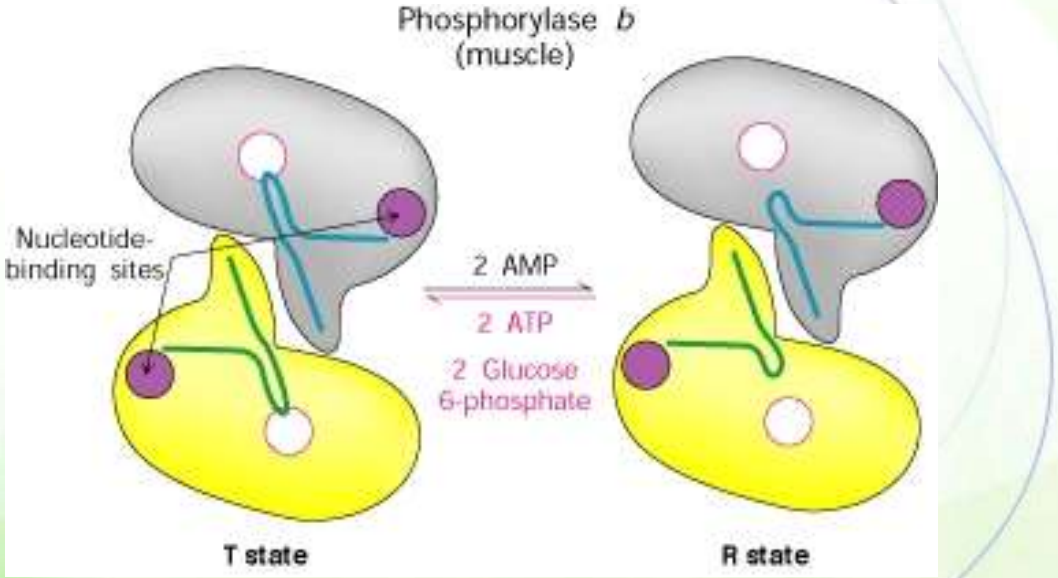


The transition of phosphorylase b between the T and the R state is controlled by the energy charge (ATP and AMP) of the muscle cell and the availability of glucose-6-phosphate.

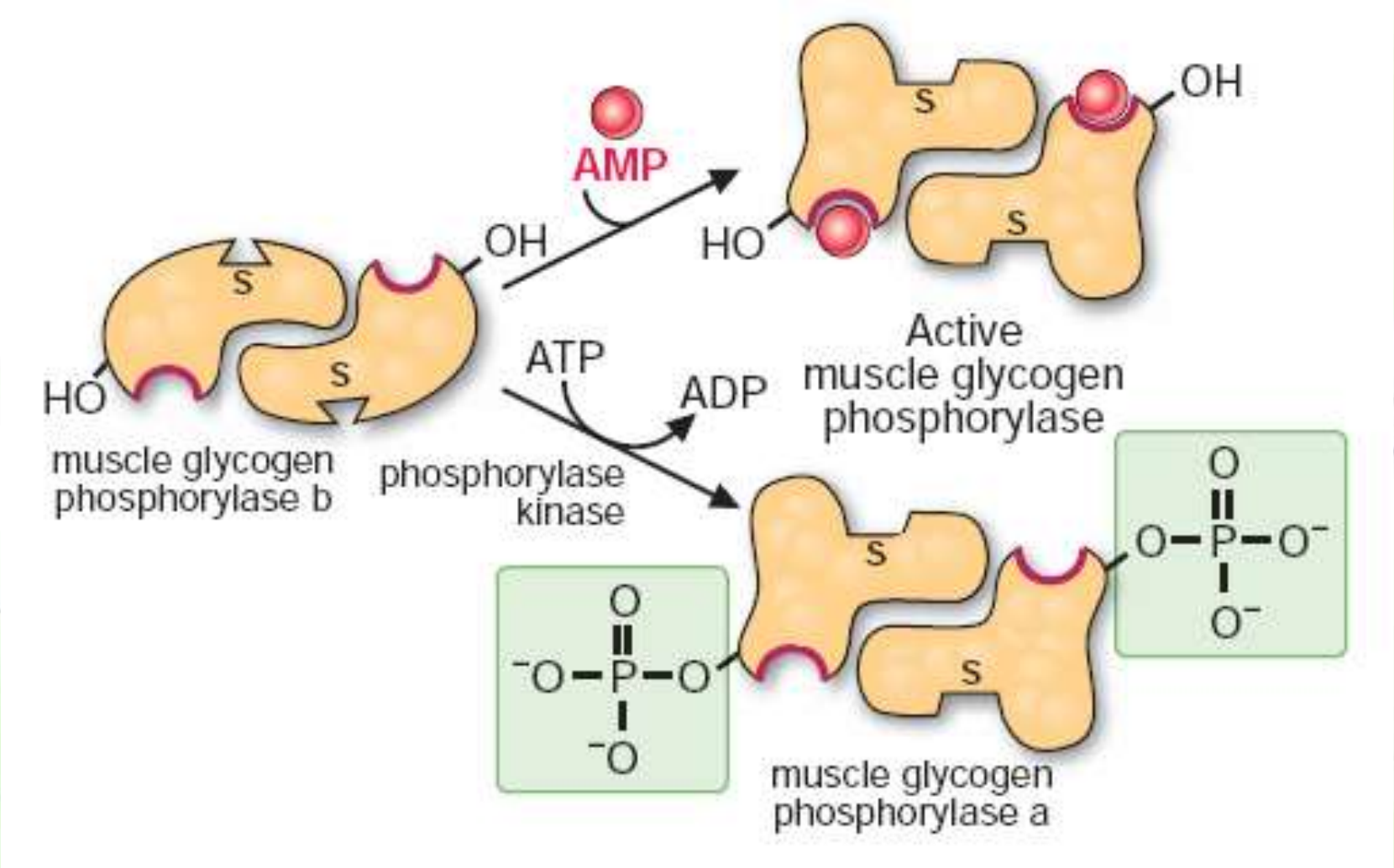
What do ATP and AMP do?

- Muscle phosphorylase b is active only in the presence of high concentrations of AMP, which binds to a nucleotide-binding site and stabilizes the conformation of phosphorylase b in the R state.
- ATP acts as a negative allosteric effector by competing with AMP and so favors the T state.

Glucose 6-phosphate also favors the T state of phosphorylase *b*, an example of feedback inhibition.



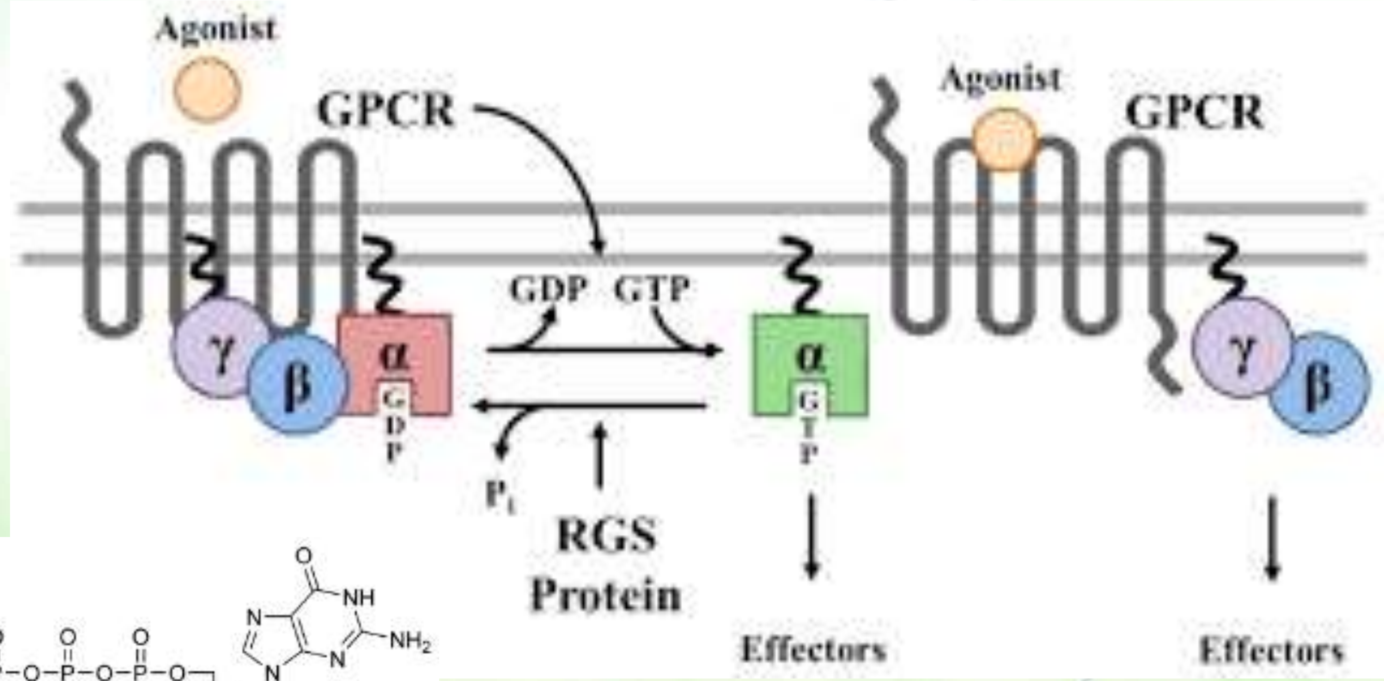
Or phosphorylate glycogen of phosphorylase b

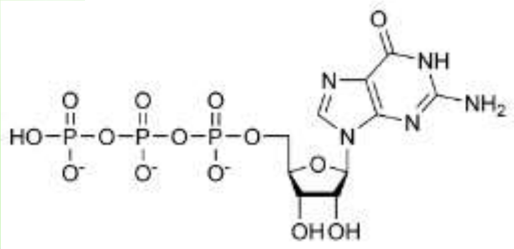


Large and small regulatory modulators

Trimeric large G proteins

- Trimeric G proteins: a family of membrane-bound proteins causing changes inside the cell. They communicate signals from hormones, neurotransmitters, and other signaling factors through G protein-coupled receptors (GPCRs)
  - **When they bind GTP, they are 'on', and, when they bind GDP, they are 'off'.**
  - **The α subunit binds to effectors stimulating or inhibiting them.**





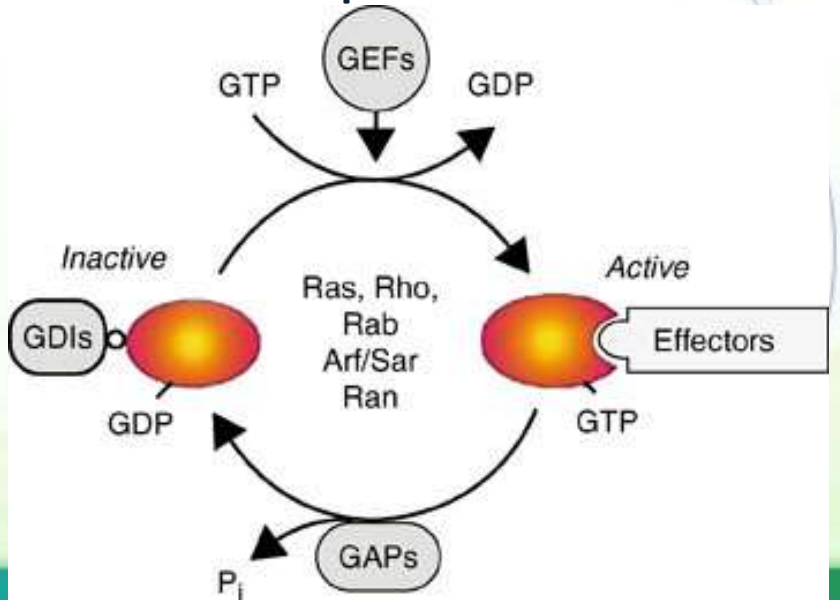
Large and small regulatory modulators

Small monomeric G proteins

- When GTP is bound, the conformation of the G protein allows it to bind target proteins, which are then activated or inhibited.
- The G protein hydrolyzes a phosphate from GTP to form GDP, which changes the G protein conformation and causes it to dissociate from the target protein.
- GDP is exchanged for GTP, which reactivates the G protein.

The activity of many monomeric G proteins is regulated by

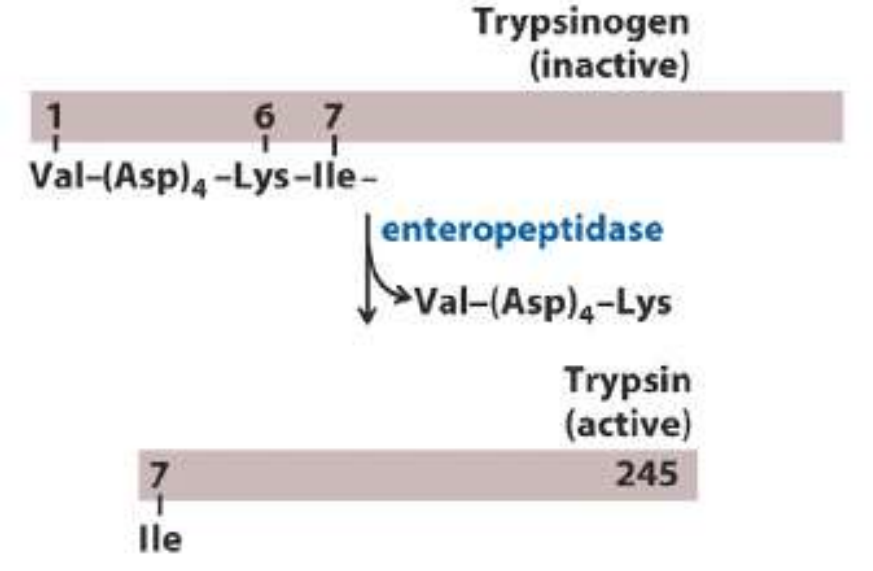
1. **GAPs [*GTPase-activating proteins*]**
2. **GEFs *[guanine nucleotide exchange factors]***
3. **GDIs [*GDP dissociation inhibitors*]**

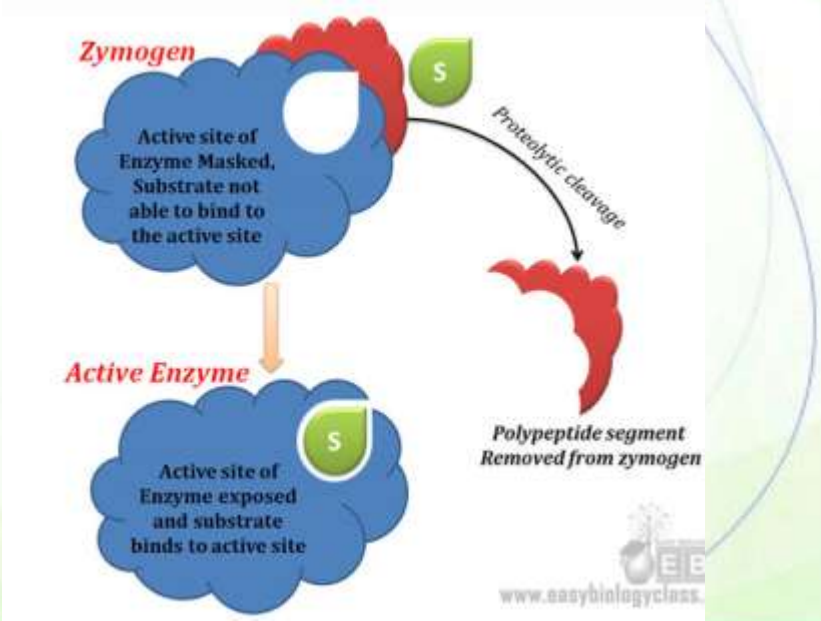


Irreversible covalent modification (Proteolytic activation)

Zymogens

- Zymogens or proenzymes are inactive precursors of enzymes.
- Activation is done by irreversibly removing part of the enzyme (usually known as the pro region present at the N-terminus).
- Examples: digestive enzymes such as chymotrypsin, trypsin, and pepsin that get activated when food is ingested.
  - Trypsinogen (zymogen) is activated via removal of the first six amino acids at the N-terminus.





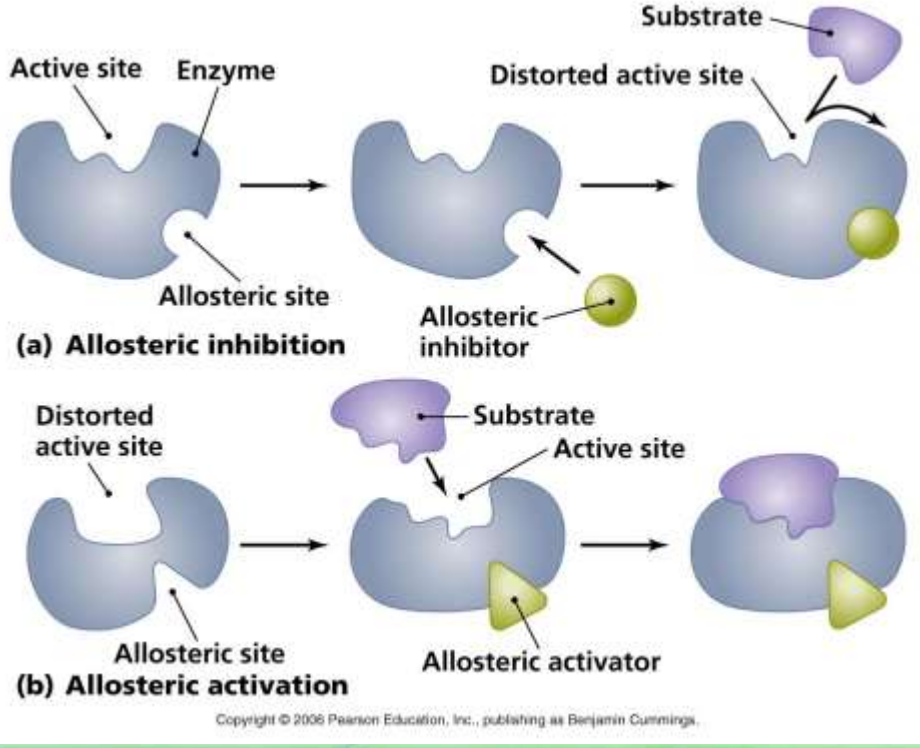
Regulation: conformational changes

- These regulatory mechanisms include
  - Allostery
  - Covalent modulation
  - Protein-protein interactions between regulatory & catalytic subunits or between two proteins;
  - Proteolytic cleavage
- Rapidly change from inactive to fully active enzyme.

Allosteric regulation

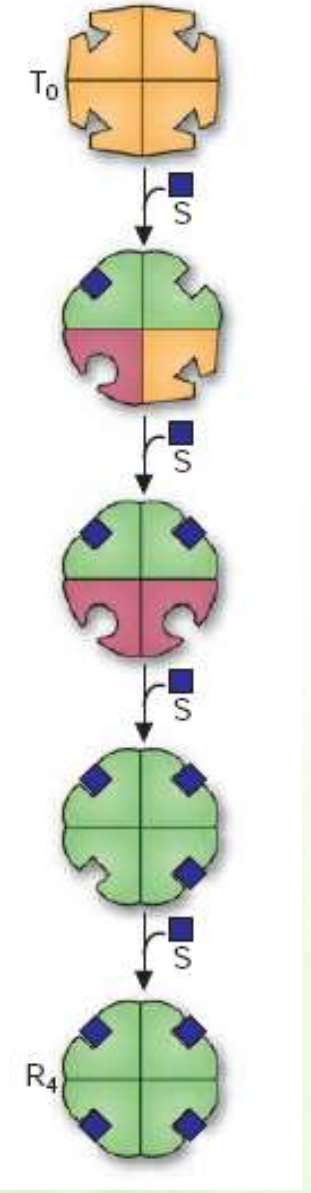
Allosteric enzymes and their modifiers

- Allosteric enzymes are multi-subunit proteins;
  - One subunit contains the active site (catalytic subunit) and another containing the regulatory site (regulatory subunit).
  - Multiple active sites can exist on multiple subunits.
- The binding of regulatory molecules triggers conformational changes in the active site via modifying non-covalent interactions.
- Allosteric enzymes bind modifiers at the allosteric site, a site that is physically separate from the catalytic site.
  - A negative allosteric modifier (inhibitor) causes the enzyme to have less activity.
  - A positive allosteric modifier (activator) causes the enzyme to be more active.



More on modifiers

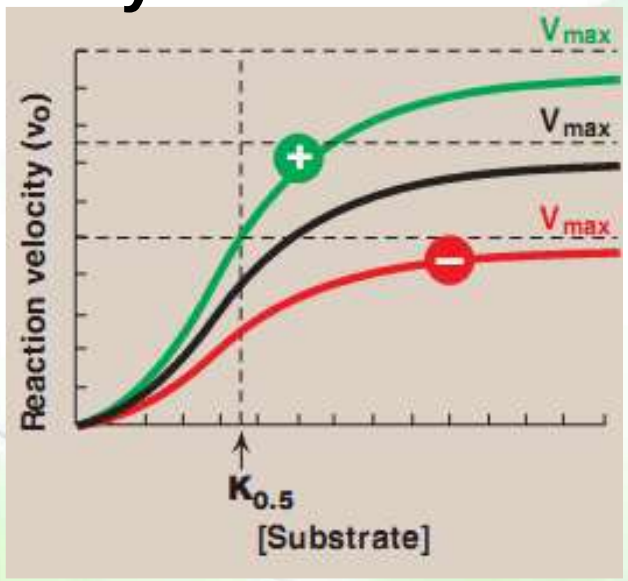
- When the modifier is a molecule other than the substrate, then it is known as heterotropic.
- If the modifier is same as the substrate, then it called homotropic.
  - The binding of the substrate causes the enzyme to become more active and binds to a second substrate at a different active site with more ease.
  - This is called "positive cooperativity”.
  - T to R conformation
  - There is also negative cooperativity.



Types of allosteric enzymes

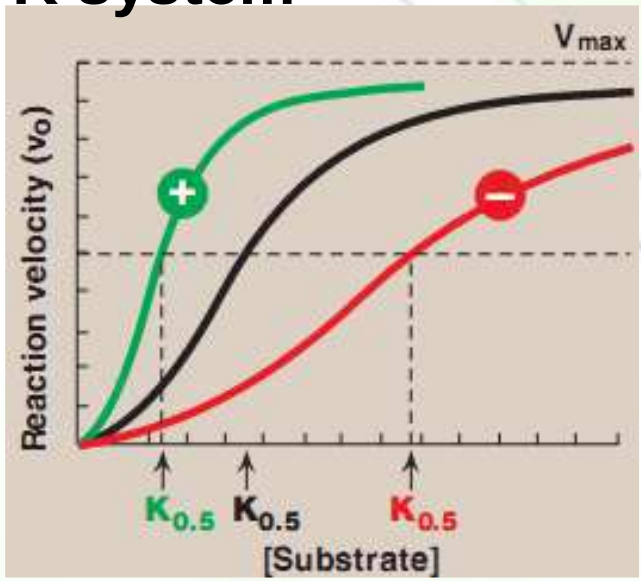
- The Michaelis-Menten model cannot explain the kinetic properties of allosteric enzymes.
- $K_{0.5}$ is used instead of K_M.

V system



Same K0.5, Different Vmax.

K system



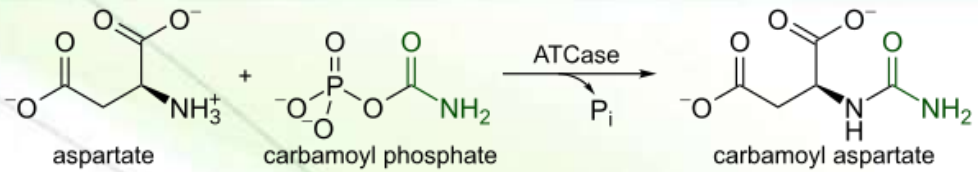
Different K0.5, same Vmax.

Note near-hyperbolic plot with activators

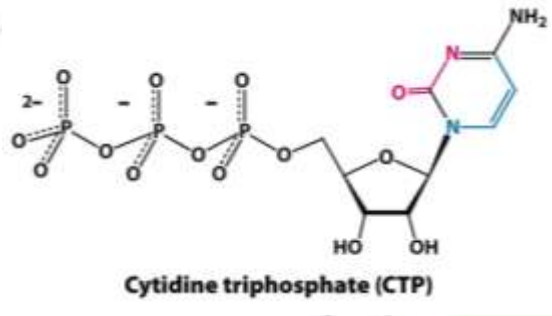
Allosteric enzymes and metabolism

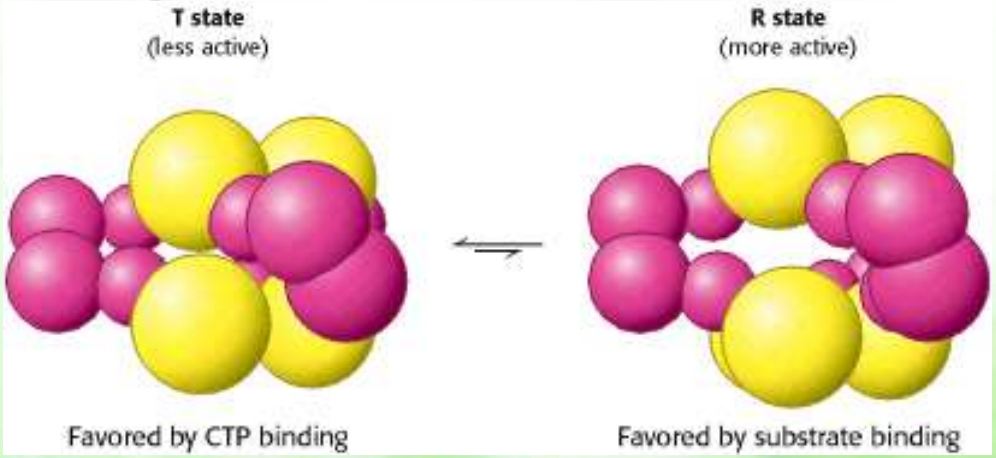
- Allosteric inhibitors usually have a much stronger effect on enzyme velocity than competitive and noncompetitive inhibitors.
- Allosteric enzymes are not limited to regulation through inhibition whereby allosteric effectors may function as activators.
- The allosteric effector needs not bear any resemblance to substrate or product of the enzyme.
- The effect of an allosteric effector is rapid occurring as soon as its concentration changes in the cell.
  - Feedback regulation of metabolic pathways by end products or by signal molecules that coordinate multiple pathways.

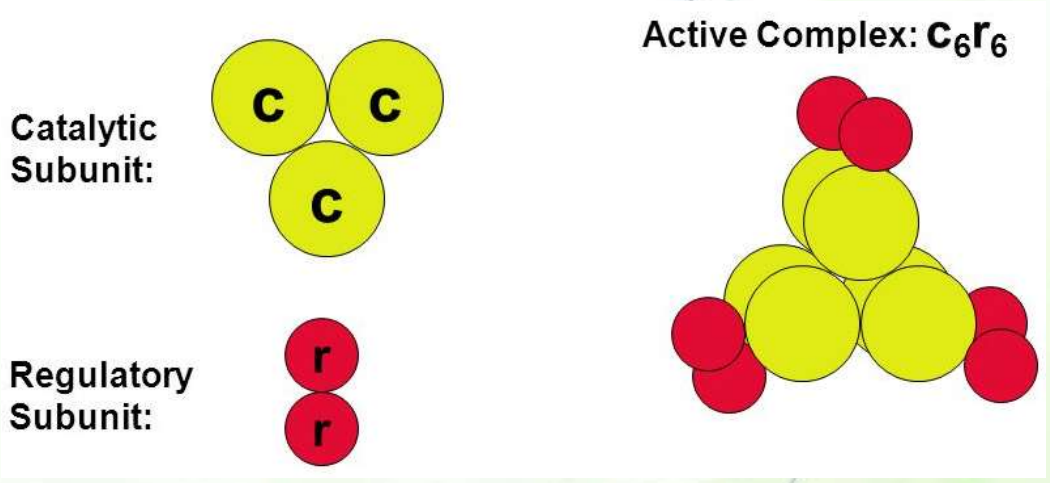
Aspartate transcarbamoylase



- Aspartate transcarbamoylase (ATCase) catalyzes the first step in the synthesis of pyrimidine nucleotides.
- ATCase consists of 12 polypeptide chains: six catalytic subunits (two trimers) and six regulatory subunits (three dimers).
- It exists in two forms: T state (less active) and R state (more active).

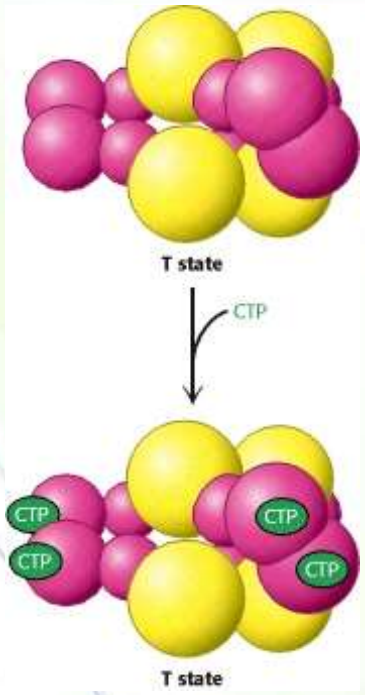


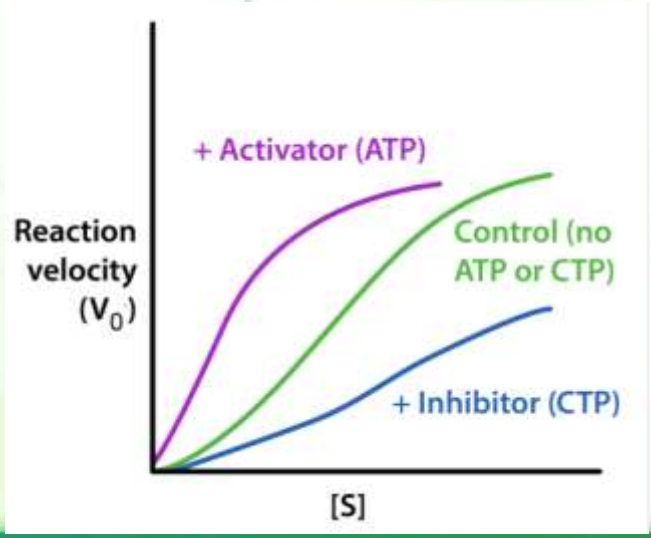




Aspartate transcarbamoylase-regulation

- ATCase is inhibited by CTP, the end-product
  - inducing a major rearrangement of subunit positions
  - stabilizing the T state of the enzyme.
  - decreasing binding affinity for Asp (substrate) at active sites on catalytic subunits
  - increasing K0.5 (K system)
    - Note: a non-competitive inhibitor changes K0.5
    - On the other hand, ATP, a purine, heterotypically activates the enzyme in order to balance the rate of synthesis of purines and pyrimidines in cells.

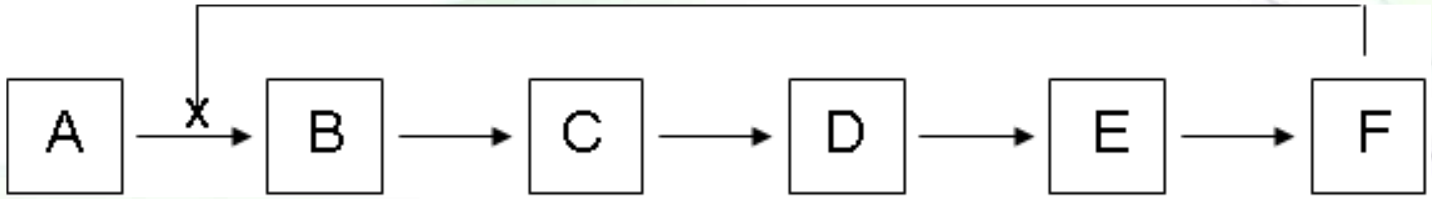


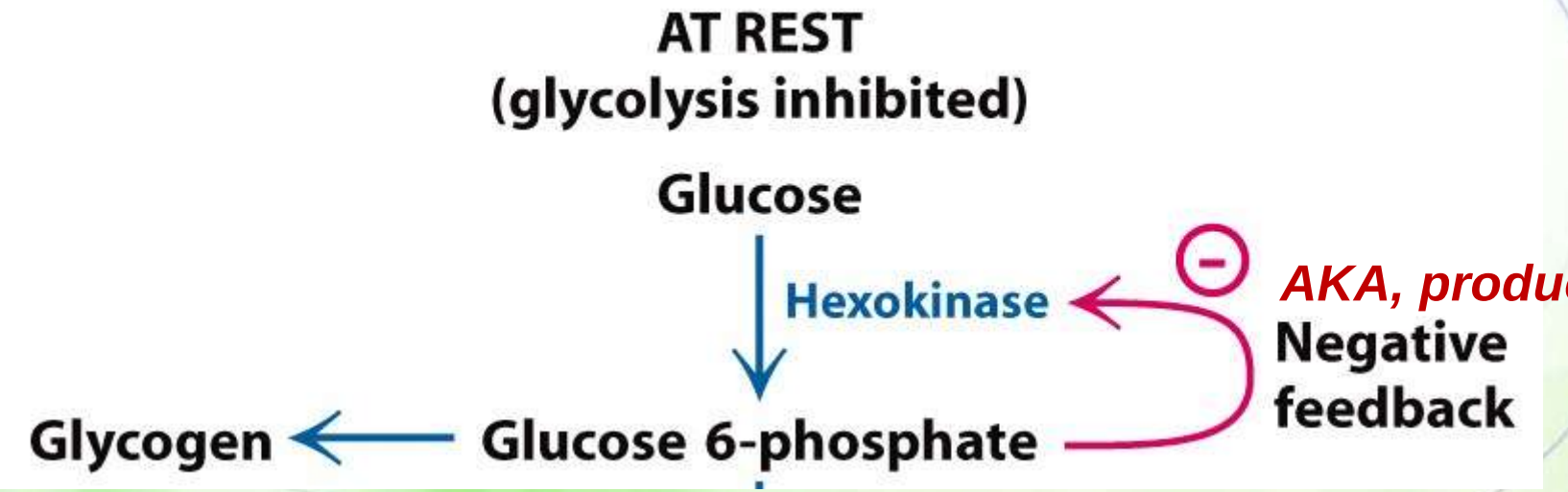


Modes of metabolic regulation

Feedback inhibition

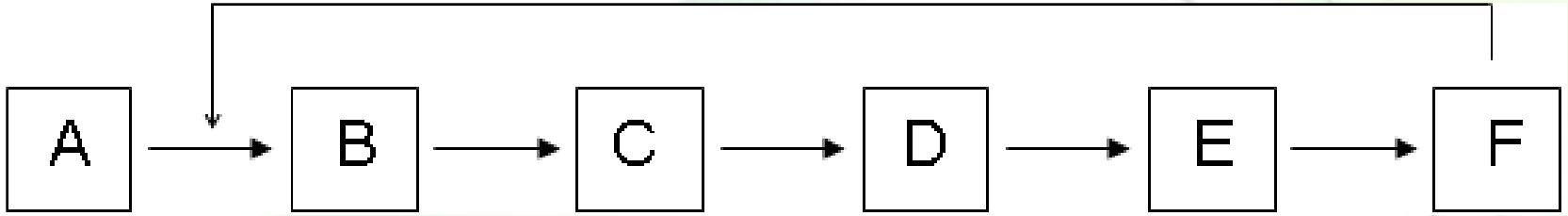
- Feedback inhibition or negative feedback regulation: an enzyme present early in a biochemical pathway is inhibited by a late product of pathway.

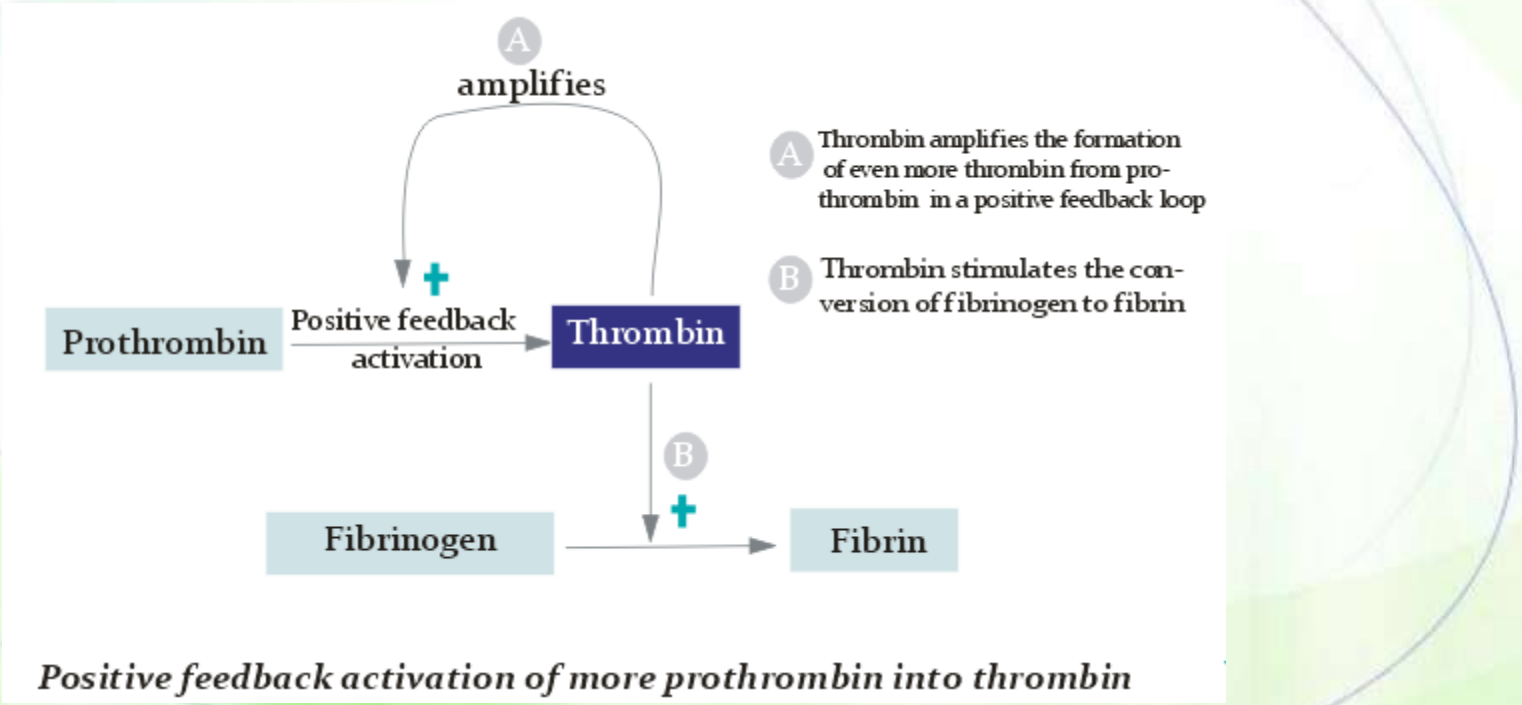




Feedback activation

- Positive feedback regulation: a product stimulates the activity of an enzyme.

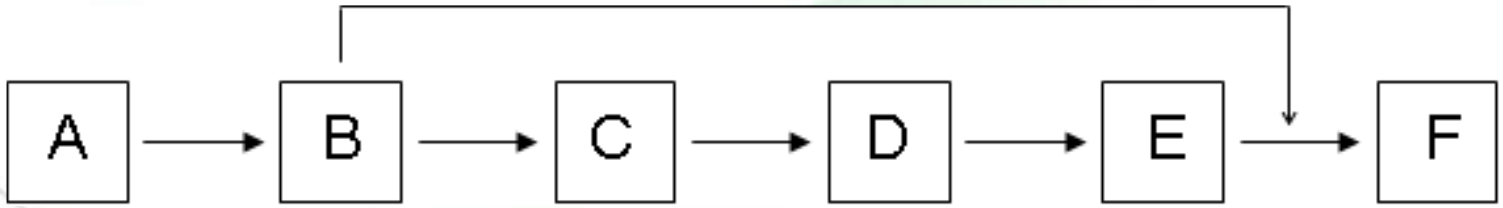




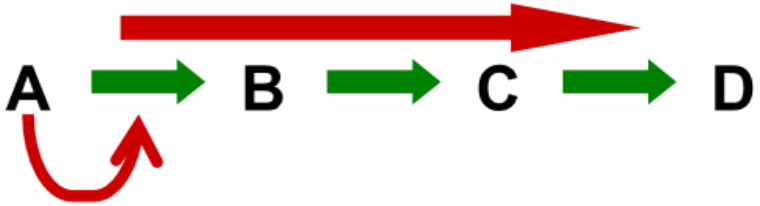
Positive feedback activation of more prothrombin into thrombin

Feed-forward activation

- Feed-forward regulation: a substrate produced early in a pathway activates an enzyme downstream of the same pathway.

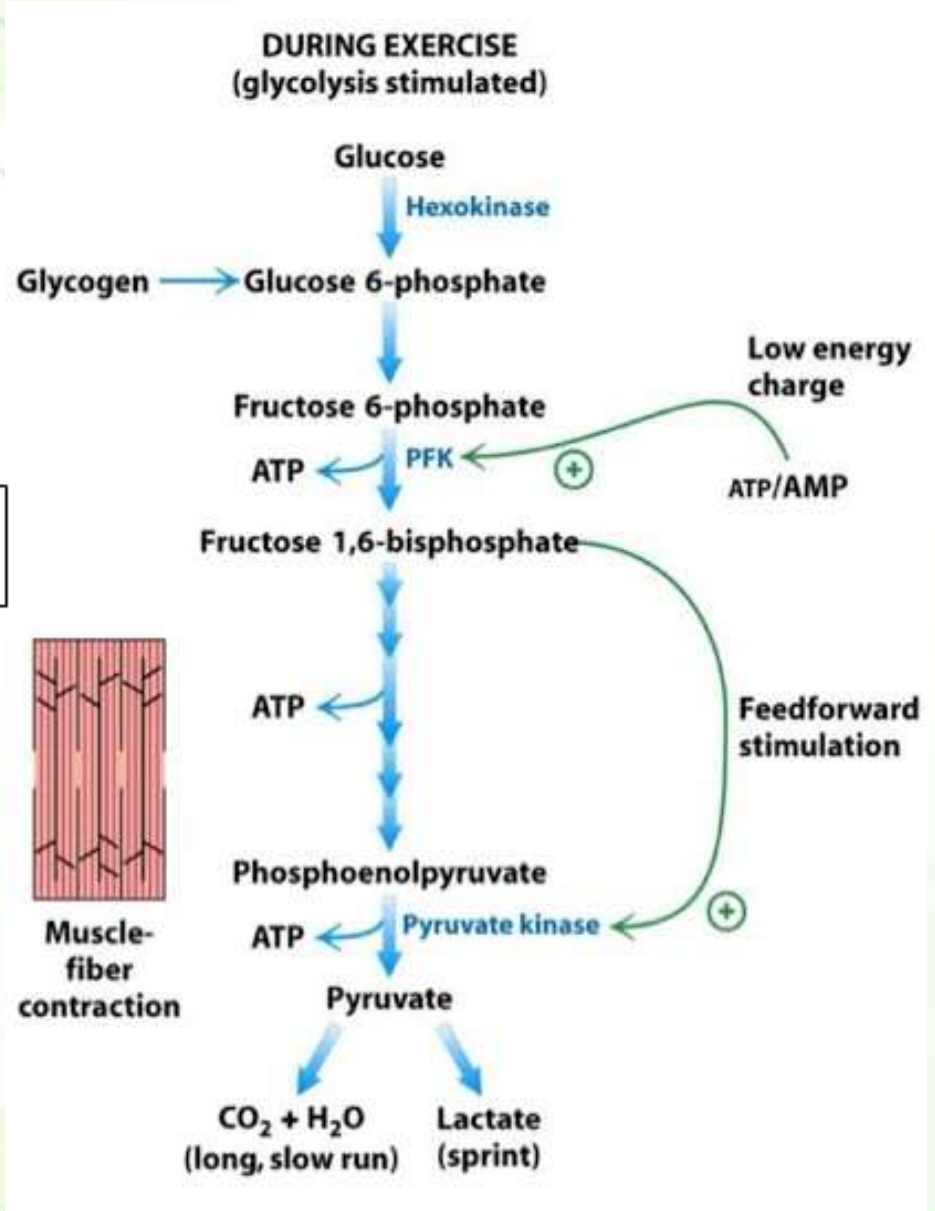


  - Glycolysis
  - Poisoning



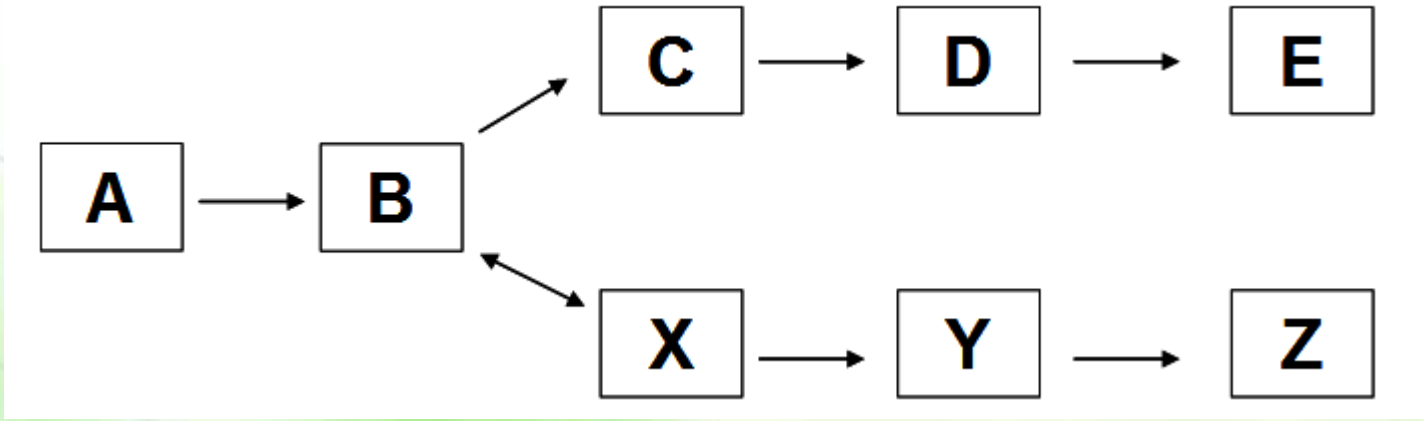
"Oh no! Get this poison out of here!"

"Alot more substra coming through!"

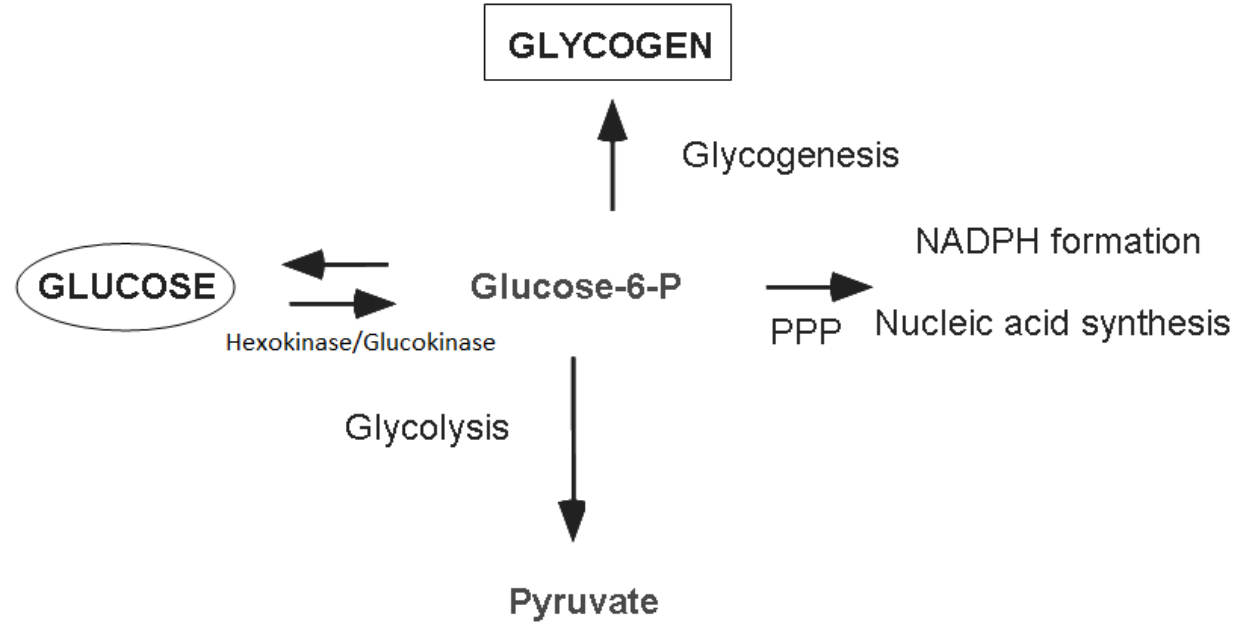


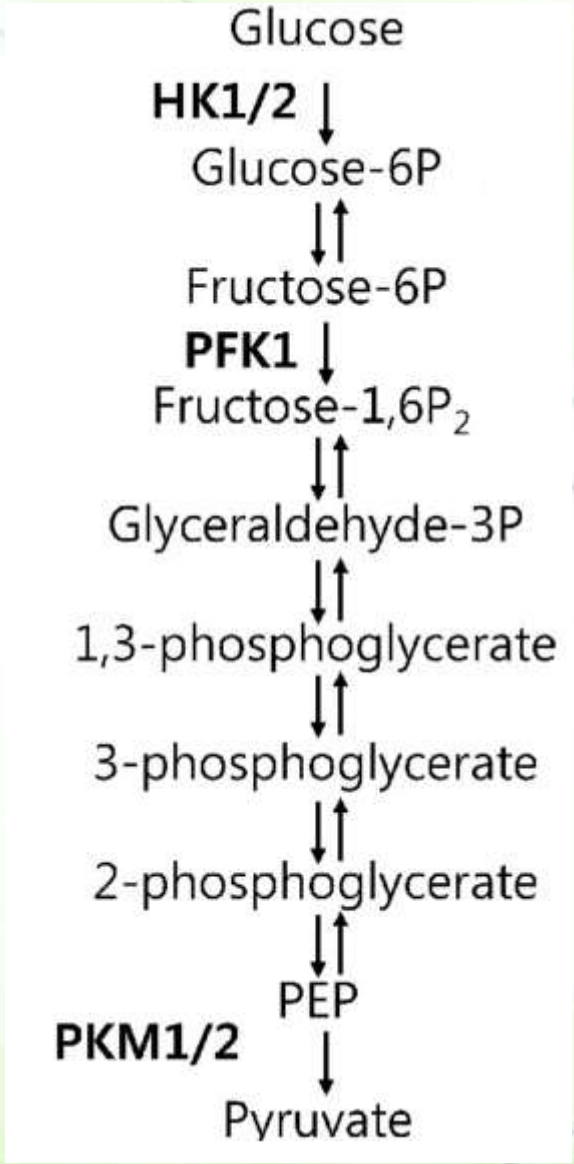
A committed step

- A committed step in a metabolic pathway is the first irreversible reaction that is unique to a pathway and that, once occurs, leads to the formation of the final substrate with no point of return.
- Committed steps are exergonic reaction.
- For example, the committed step for making product E is (B → C), not (A → B).



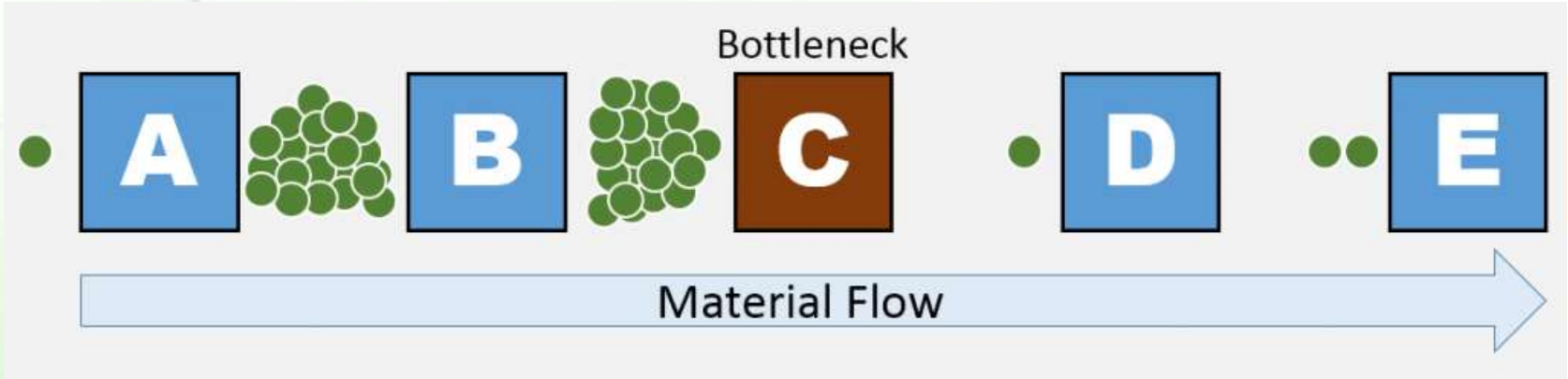
PFK, not HK/GK, is the committed step





Rate-limiting reactions

- Rate-limiting reactions slow down rate of reactions because:
  - requirement for high amount of energy
  - strict regulation of enzymes
  - high Km values of enzyme towards its substrate
- These reactions are also usually, but not necessarily, committed steps.



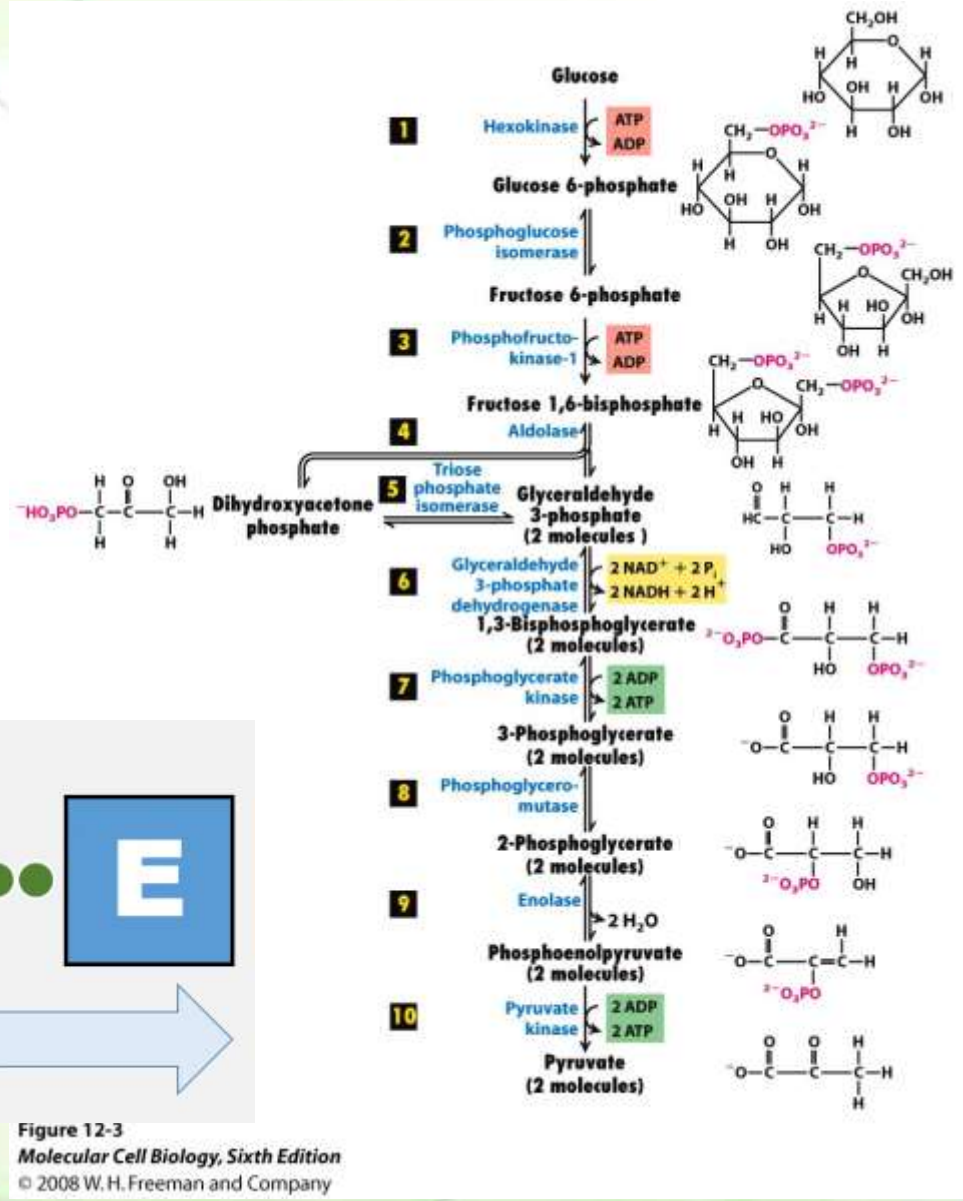


Figure 12-3
Molecular Cell Biology, Sixth Edition

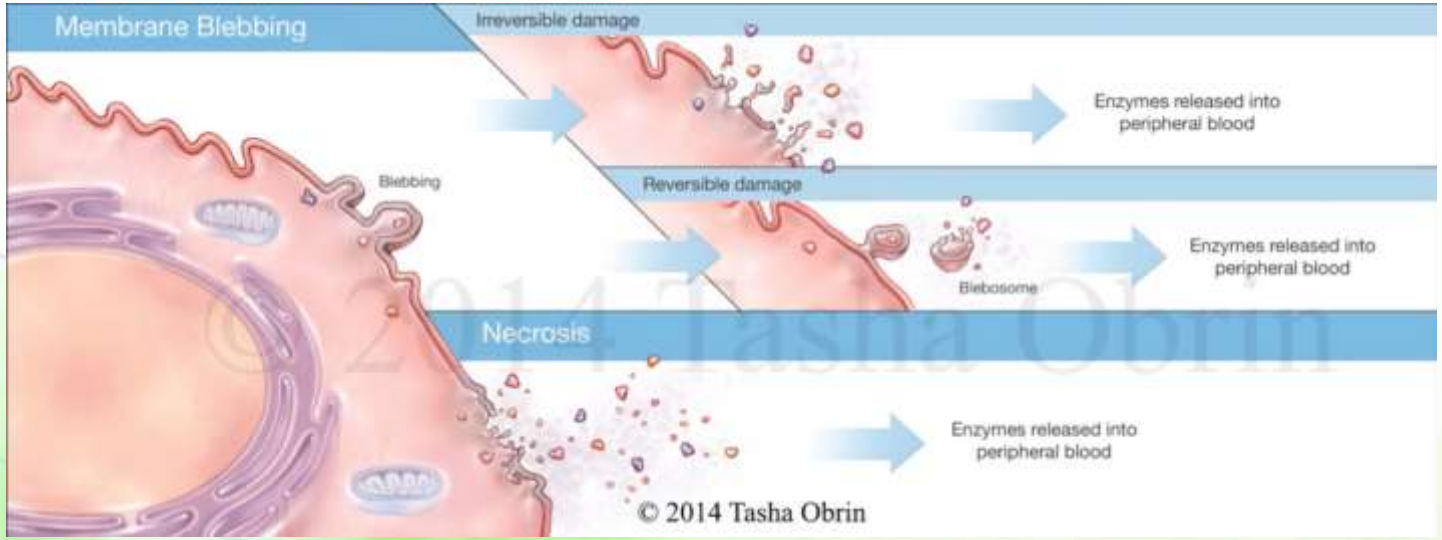
Enzymes in disease diagnosis

Concept

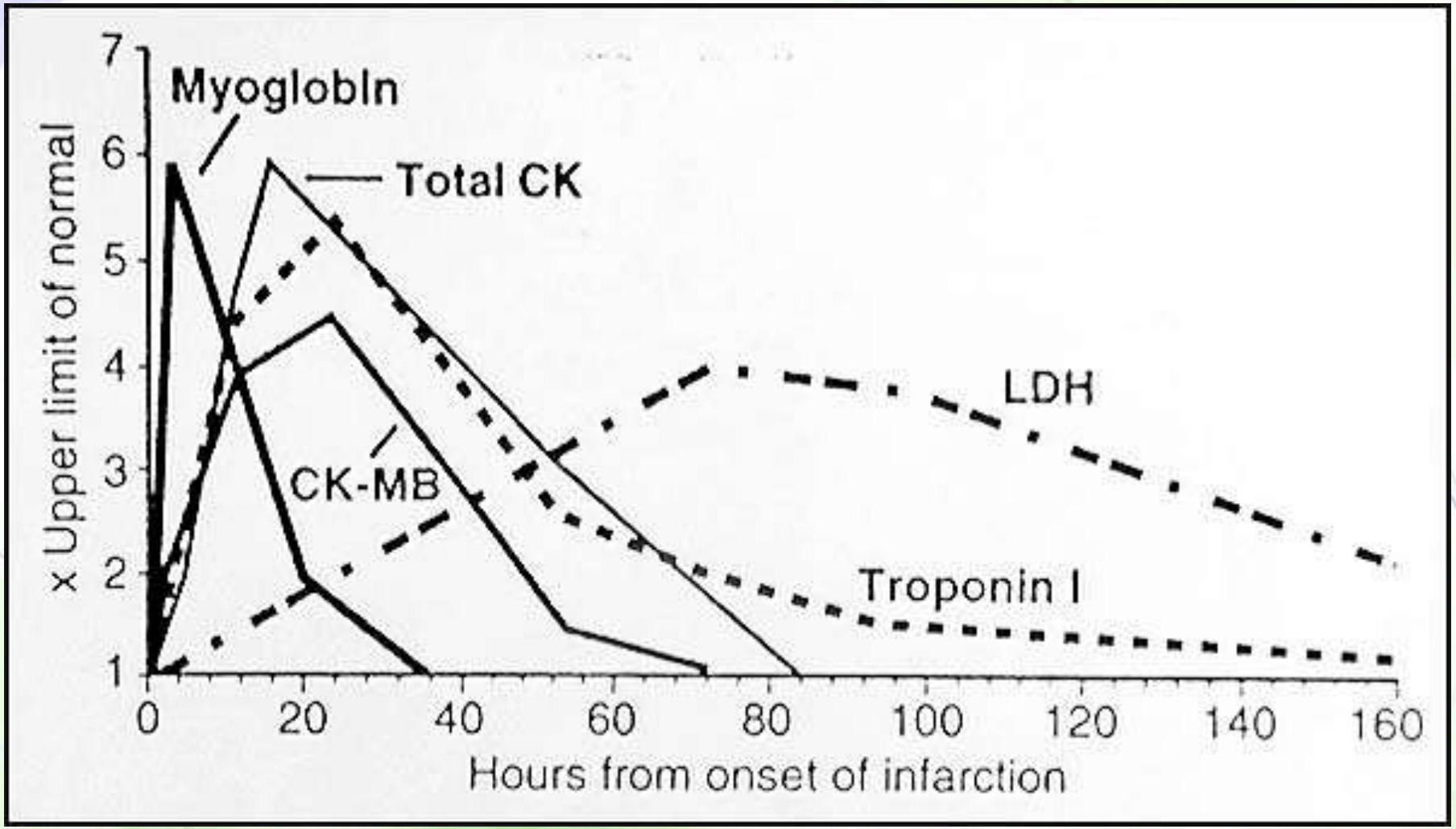
- The presence of enzymes in serum indicates that tissue or cellular damage.
- The measurement enzyme amount in serum is of diagnostic significance.
- Examples:
  - The amino transferases: alanine transaminase, ALT and aspartate aminotransferase, AST
  - Lactate dehydrogenase, LDH
  - Creatine kinase, CK (also called creatine phosphokinase, CPK)

AST and ALT

- The typical liver enzymes measured are AST and ALT.
- ALT is predominantly in hepatocytes.
- The ratio of ALT/AST is diagnostic.
  - Liver disease/damage (not of viral origin) < 1.
  - Viral hepatitis > 1.

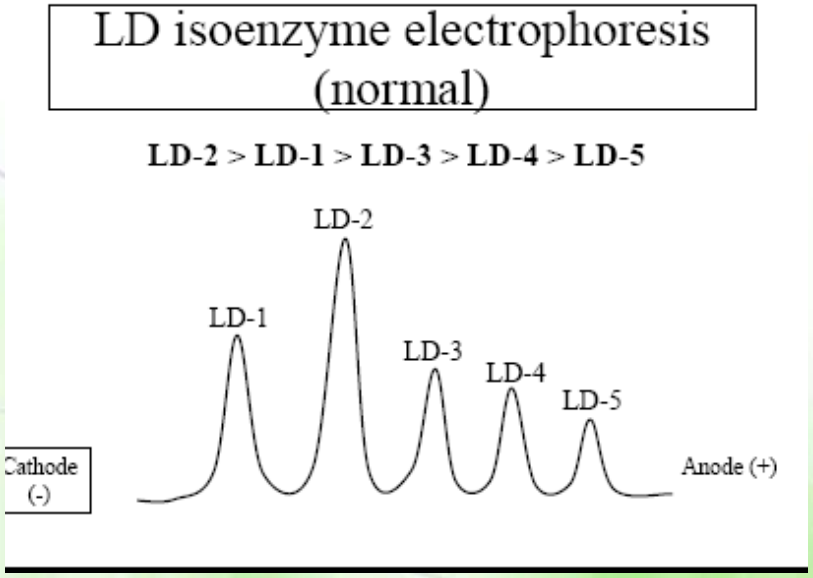


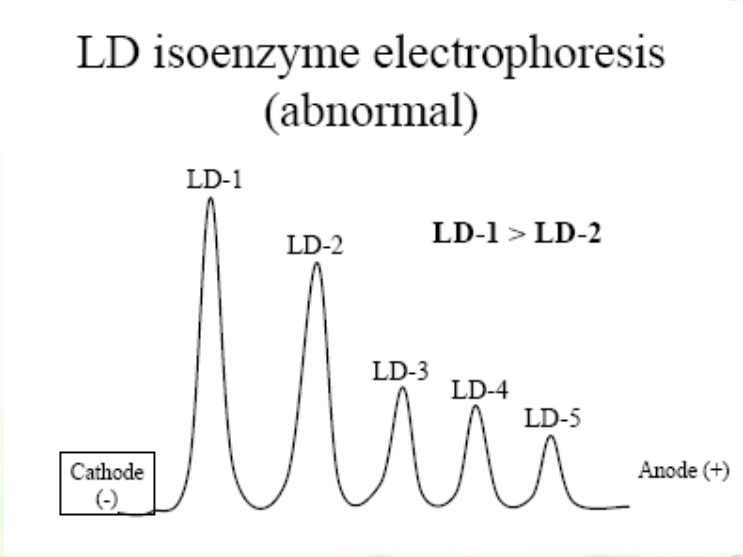
Protein profile in myocardial infarction



LDH

- A comparison of serum levels of LDH-1/LDH-2 ratio is diagnostic for myocardial infarction (heart attacks).
- Normally, this ratio is less than 1.
- Following an acute myocardial infarct, the LDH ratio will be more than 1.





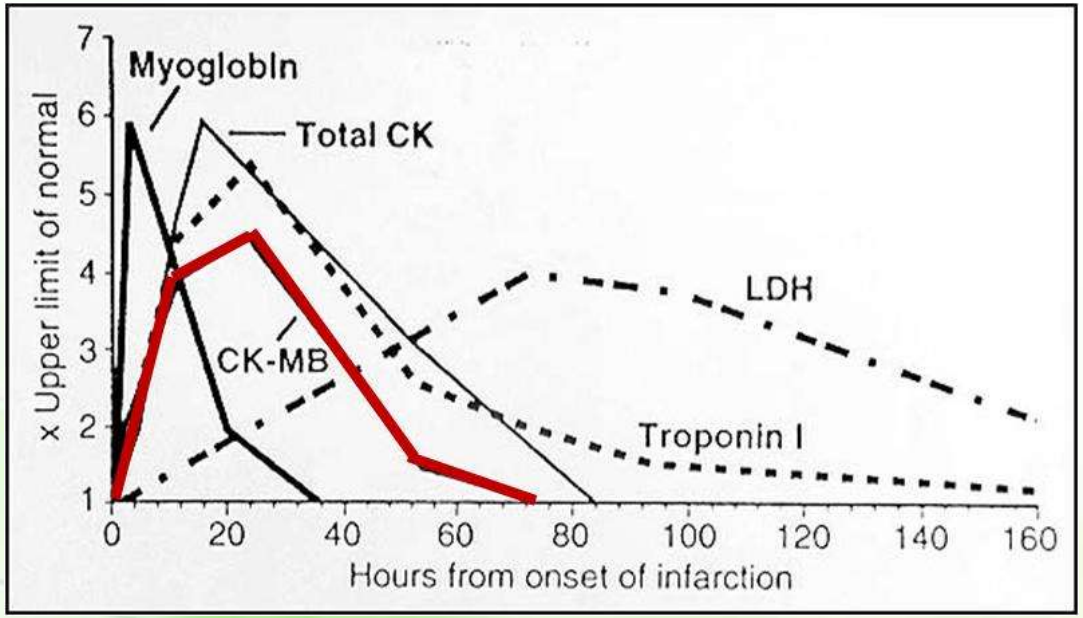
CPK

- CPK is found primarily in heart and skeletal muscle as well as the brain.
- Three tissue-specific isozymes of CPK:
  - CPK3 (CPK-MM)
  - CPK2 (CPK-MB)
  - CPK1 (CPK-BB)

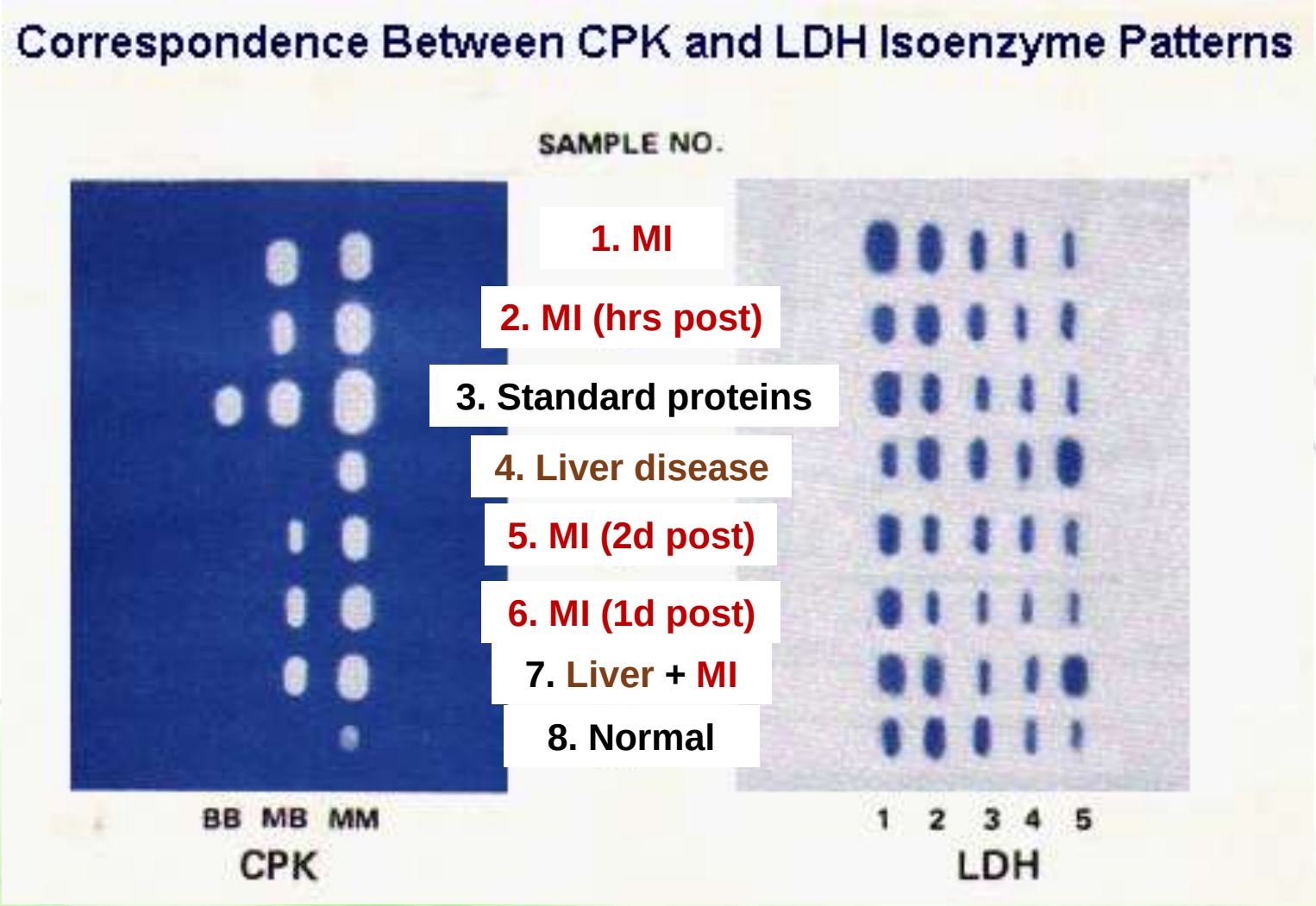
Serum	Skeletal Muscle	Cardiac Muscle	Brain
0 trace BB <6% MB >94% MM	0 trace BB 1% MB 99% MM	0% BB **20% MB** **80% MM**	97% BB 3% MB 0%MM

CPK and myocardial infarction

- A significant amount of CPK-MB is released after MI leading to increased CPK-MB/total CPK ratio (diagnostic of an acute infarction).
  - An increase of total CPK in itself may not.
- CPK-MB disappears in 1-3 days, so another elevation is indicative of another event (reinfarction).



Example


Correspondence Between CPK and LDH Isoenzyme Patterns
SAMPLE NO.
1. MI
2. MI (hrs post)
3. Standard proteins
4. Liver disease
5. MI (2d post)
6. MI (1d post)
7. Liver + MI
8. Normal
BB MB MM
CPK
1 2 3 4 5
LDH

Interpretation

- Sample #3 represents results for standard proteins.
- Sample #8 results are from a normal specimen.
- Sample# 1 MI patient. The specimen was collected at a time when the activity of both LDH and CK were elevated. Note the LDH flip and the high relative activity of the MB isoenzyme.
- Sample# 2 MI patient who experienced chest pain only several hours previously. Total CK is significantly elevated.
- Sample# 6 MI patient (the 1st day post MI); CK level is elevated with a high relative MB isoenzyme activity and the LDH flip is evident.
- Sample# 5 MI patient (2 days post MI) is like sample #6, but lower CK levels.
- Sample# 7 MI patient with passive liver congestion or the patient was involved in an accident as a consequence of the MI, and suffered a crushing muscle injury.
- Sample# 4 a patient with liver disease.

Troponins in MI

- Troponin levels rise within four to six hours after the beginning of chest pain or heart damage and stay elevated for at least one week.
- This long elevation allows detection of a myocardial infarction that occurred days earlier but prevents detection of a second infarction if it occurred only days after the first.