

METABOLOMICS

Exam: written or oral, 3 questions to describe metabolic pathways (1 part) and 2 about metabolomics analysis (2part)

- 1-2)introduction
- 3)metabolism: an overview
- 4)chemical reactions in cells
- 5)chemical reactions in cells
- 6)enzymology and glycolysis
- 7)glycolysis and gluconeogenesis
- 8)glucose storage metabolism
- 9)pentose phosphate pathway
- 10)aerobic fate of pyruvate-the tca cycle
- 11)tca cycle regulation and fatty acid degradation
- 12)ketone bodies
- 13)aminoacids catabolism
- 14)oxidative phosphorylation
- 15)oxidative phosphorylation
- 16)photosynthesis
- 17)general workflows in metabolomics
- 18)mass spectrometry
- 19)nitrogen metabolism and amino acids and nucleotides
- NMR lecture
- 20)fatty acids synthesis
- 21)lipid synthesis and data analysis
- 22)data analysis and introductory lesson on opa1 metabolomics experiment
- 23)fluxomics
- 24)examples of metabolomics and fluxomics applications

1/2-Introduction

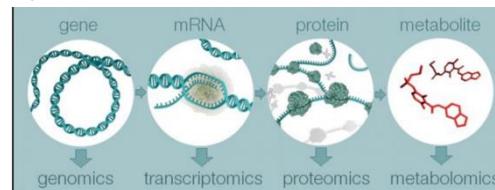
What is metabolomics?

>A field of life science research that uses High Throughput (HT) technologies to identify and/or characterize all the small molecules or metabolites in a given cell, tissue or organism (i.e. the metabolome).

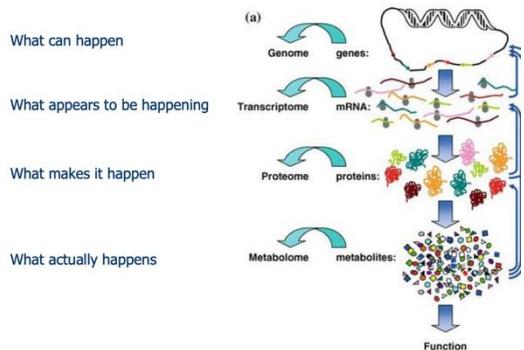
Metabolomics : take a picture of the composition of our metabolites

Metabolomics is one of the more recently introduced “omics” technologies

Proteins are important to produce metabolites



Metabolomics try to quantify the phenotype



When we study the genome, we know what can happen (genes don't express at the same time, different cells can express different genes)

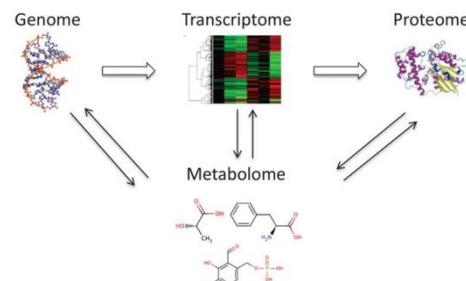
When we study transcriptomics, we know what appears to be happening (genes are expressed but not mRNAs are translated)

Proteomics: we know what proteins can do, we can foresee what they will do, we can have this info only performing metabolomics

Metabolomes create the phenotype

The metabolome is connected to all other “omes”

They are strictly interconnected (nitrogen bases (metabolites) are the building blocks of nucleic acids). Some metabolites can influence the expression of our genes (methylation depends on metabolites). Proteome also influenced by metabolome (several metabolites are activators/inhibitors in the activity of enzymes, aa building blocks of proteins)



Metabolomics enables system biology

The study of all these omics sciences is called systems biology: all this info together to have a picture of the entire biological system

What is a metabolite?

>Any organic molecule detectable in the body with a MW(molecular weight) < 1500 Da

>Includes peptides, oligonucleotides, sugars, nucleosides, organic acids, ketones, aldehydes, amines, amino acids, lipids, steroids, alkaloids, foods, food additives, toxins, pollutants, drugs and drug metabolites

>Include human and microbial products

>Metabolites are the organic molecule that you can analyze in small and large samples> usually the techniques are able to detect a minimum detectable concentration of 1pM

What is a metabolome?

>The complete collection of small molecule metabolites in a cell, tissue or organism

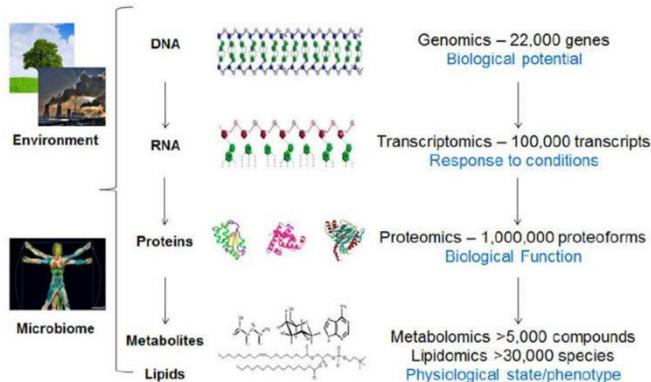
>Includes endogenous and exogenous molecules

>Up to now is not possible to extract and identify all the metabolites in an unique extraction and detection technology.

Because there are very different molecules > techniques cannot analyze all these metabolites in only one experiment. So we have to use different methods with different solvents for different molecules (like hydrophobic and hydrophilic)

>Metabolome size is ill-defined (not completely defined)

How many metabolites?

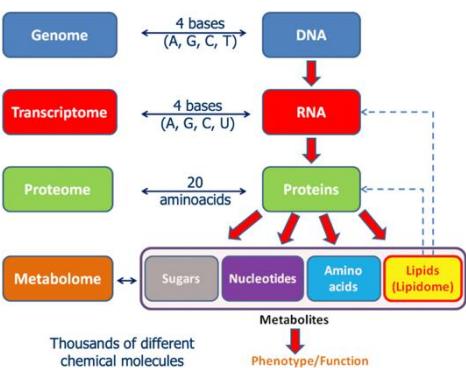


>it is estimated that all plant species contain 90,000-200,000 compounds

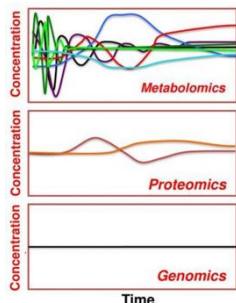
>Each individual plant species contains about 5,000-30,000 compounds

>Up to date, the number of metabolites in human metabolome is estimated in 100,000 compounds, but is far from complete

Why is metabolomics difficult?



Because the molecules are very different from chemical point of view and also the metabolome can change over time



- >Concentrations of cellular metabolites vary over several orders of magnitude (mM to pM)
- >Differences in molecular weight (20-2000 Da)
- >High turnover rates
- >Some metabolites are labile

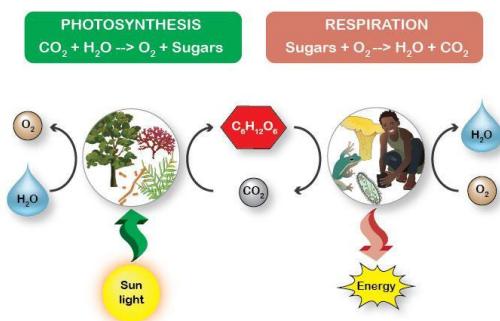
Why is metabolomics important?

- >95% of all diagnostic clinical assays test for small molecules (Clinical tests > performing target metabolomics, we detect the presence of specific organic molecules)
- 89% of all known drugs are small molecules (drugs can modify our metabolome)
- 50% of all drugs are derived from pre-existing metabolites
- 30% of identified genetic disorders involve diseases of small molecule metabolism
- Small molecules serve as cofactors and signaling molecules to 1000's of proteins

There are many applications of metabolomics: drug discovery, microbiology, drug evaluation, systems biology, nutrition, clinical biomarkers, plant technology

3-Metabolism overview

Autotrophs and heterotrophs organisms



>Autotroph organisms:

- Photosynthetic organisms: energy from sunlight to produce sugars
- Other organisms using redox reactions can extract energy from sugars

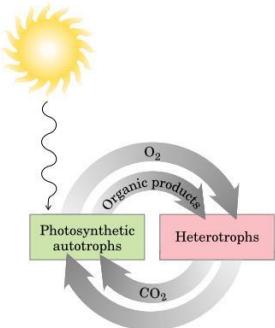
>Heterotroph organisms:

- able to intake organic molecules and degrade them extracting the energy for the reactions important for life

Photosynthesis: carbon dioxide plus water used to produce glucose and oxygen

Respiration is the opposite of photosynthesis

Cyclin of carbon



- Carbon is present in atmosphere in the most oxidized form =carbon dioxide
- Carbon dioxide can be used in photosynthesis to produce sugars
- the cycle of carbon is continuous with heterotroph organisms in which sugars are degraded to form carbon dioxide

FIGURE 1 Cycling of carbon dioxide and oxygen between the auto-trophic (photosynthetic) and heterotrophic domains in the biosphere. The flow of mass through this cycle is enormous; about 4×10^{11} metric tons of carbon are turned over in the biosphere annually.

Cyclin of nitrogen

- Nitrogen =another important element of organic molecules
- The cycle of nitrogen starts with atmospheric nitrogen, but only few organisms are able to fix nitrogen inside organic molecule (they intake nitrogen and bind it inside organic m.)
- The organisms able to fix atmospheric nitrogen= nitrogen fixing bacteria (the fixing reaction requires a lot of energy, because nitrogen is not reactive)
- After fixation nitrogen is reduced to form ammonia, which can be absorbed by some organisms: PLANTS can, animals cannot.
- Also nitrifying bacteria can absorb ammonia: they nitrify ammonia so that plants can absorb it
- Animals can intake ammonia only be eating plants, then nitrogen can enter as aa but also as nitrogen bases. Animals have a metabolism that try to spare or recycle nitrogen, because animal cells cannot produce it directly.
- When animals die, they are degraded by bacteria > formation of ammonia

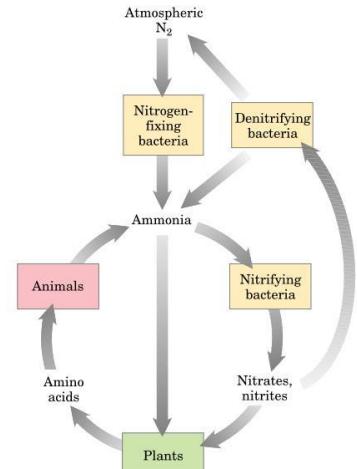


FIGURE 2 Cycling of nitrogen in the biosphere. Gaseous nitrogen (N_2) makes up 80% of the earth's atmosphere.

Metabolism: an overview

- >The word metabolism comes from the Greek *metabolé* and means change or transformation.

> This conversion or transformation of chemicals involves a large number of chemical reactions with many chemical intermediates, the completeness of these reactions is called METABOLISM, and the chemicals involved in metabolism are called METABOLITES.

- Each point corresponds to a metabolite and each line to a reaction

- Reactions are always catalyzed by enzymes

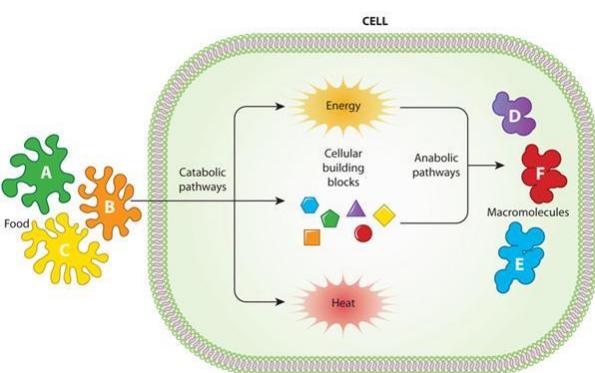
- All the metabolisms are interconnected to each others

> Metabolism is the sum of all the chemical transformations taking place in a cell or organism.

> Transformations occur through a series of enzyme-catalyzed reactions that constitute metabolic pathways.

> Metabolism is highly connected and forms a network in which few metabolites participate to several pathways.

- At the center of all metabolic pathways we have carbohydrate metabolism



Cellular metabolism has two main goals:

> to synthesize the building block required for growth and maintenance of cells

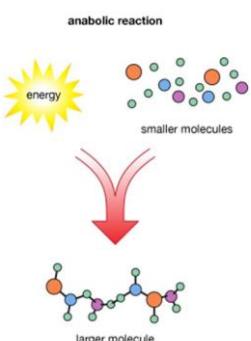
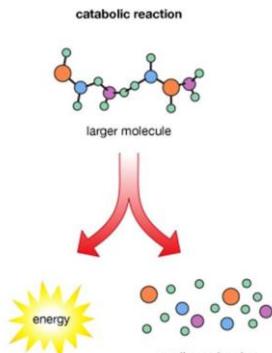
> to produce the energy necessary for vital functions of cells

Catabolism and anabolism

Metabolisms can be divided in 2 big parts:

> **catabolism** = sum of all the pathways by which cells degrade organic nutrients (food) to form simple molecules to extract chemical energy.

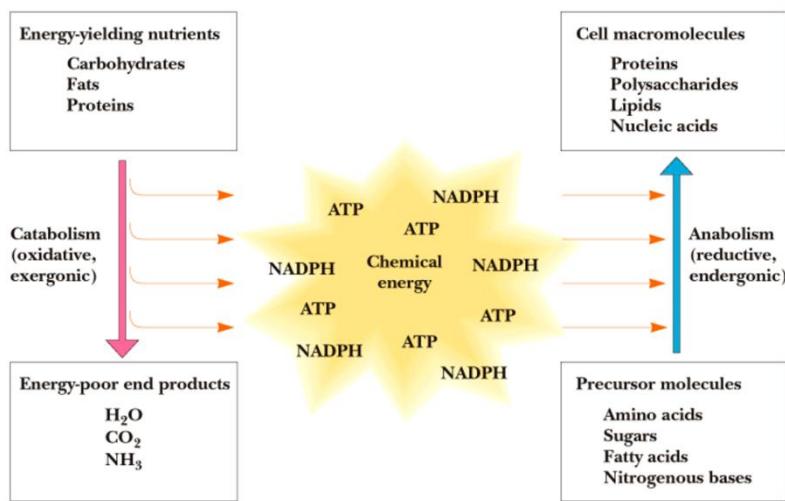
So, macromolecules are degraded in simple molecules, which are then used for production of energy and the production of new macromolecules



> **anabolism** = reactions of synthesis of NEW macromolecules from small molecules and energy

So, anabolism is the sum of pathways that start with small precursor molecules and convert them to progressively larger and more complex molecules. These pathways require the input of energy.

Because cells don't waste energy, the catabolic and anabolic pathways are strictly regulated



Catabolism and anabolism are interconnected by 2 important energetic molecules: ATP and NADPH

ATP= carrier of energy, to give energy to reaction that needs it

NADPH= or NADH, can transport electrons at high energetic levels, during catabolic reactions, we have redox in which reduced molecules are degraded (oxidative reactions)

Electrons at high energy levels are transported from oxidized molecule to NADPH, which conserves the electronical energy. So NADPH is called reducing molecule

Metabolism is organized in pathways

>Pathways are the sum of consecutive reactions converting precursors into products.

>The presence of several steps (and so the formation of intermediates) provides high flexibility and sophisticated regulation of the overall metabolism.

> Enzymes could work separately or organized in multienzymatic complexes.

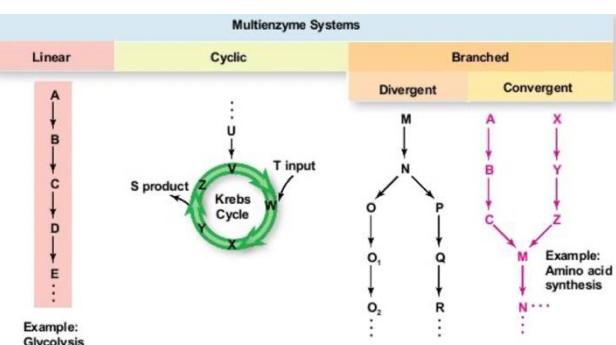
> Enzymes could be physically grouped or separated in cellular compartments

Each reaction catalyzed by enzymes can be regulated > each enzyme can be regulated > cells can regulate all the pathways

Enzymes could work separately or organized in multienzymatic complexes

Some enzymes are membrane proteins > reaction occurs in the membrane > ex : oxidative phosphorylation, also synthesis of complex lipids and elongation of fatty acids

Patterns of metabolism



Metabolic pathways are organized in different ways:

>**linear** pathways: consecutive reactions step by step in a linear frame, some intermediates can be used for other metabolic pathways

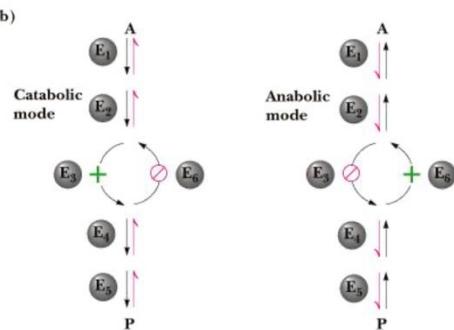
>**cyclic** pathways: characterized by the presence of intermediates transformed in a cyclic way, at the end of the reactions we have the starting intermediate. (Krebs cycle)

>**branched** pathways:

-divergent: at the beginning some common reaction, then the intermediate can be split in 2 molecules

-convergent: 2 different reactants transformed, but at the end condensation

Amphibolic pathways



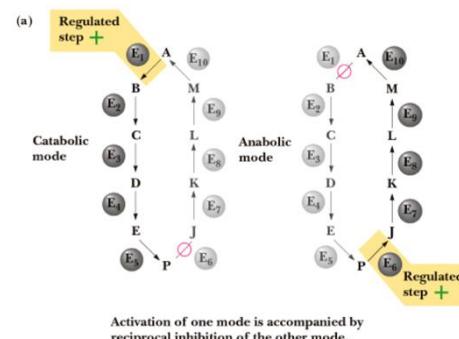
Amphibolic pathways > we have a lot of reactions in catabolic or anabolic ways catalyzed by the same enzyme. A lot of these reactions are reversible, the same enzyme can also catalyze the inverse reaction. However, in some specific points of these pathways we have one or more specific reactions catalyzed by different enzymes > usually these are irreversible reactions

These pathways can be regulated by regulating only one or two enzymes (of the irreversible reactions)

Example: regulation of glycolysis and gluconeogenesis> they both occur in cytosol, and they do not occur at the same time

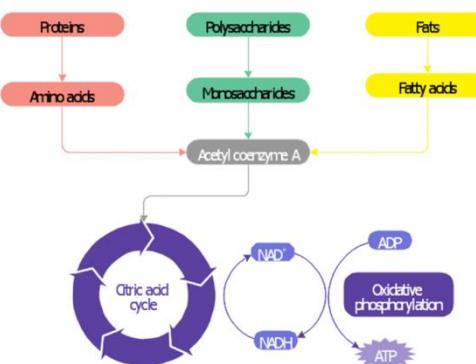
Separated pathways

Sometimes we can have not amphibolic pathways> catabolic and anabolic pathways occur using completely different enzymes. Their regulation is often at the beginning of the pathway



Metabolic pathways are compartmentalized

Catabolism schematic



example: most of the metabolic pathways for the synthesis of sugars in plants are in chloroplasts

Catabolic pathways converge to produce few molecules

Macromolecules are degraded into smaller molecules, which, when cells need energy, are oxidized to form acetyl coenzyme A= substrate for starting the citric acid cycle (Krebs cycle), which is completely oxidized to form ATP

Anabolic pathways diverge to produce a lot of molecules

Acetyl co a can be used for energetic metabolism but also for the synthesis of a lot of molecules: like cholesterol, fatty acids...

Citric acid cycle: at the crossroad between catabolism and anabolism

A lot of its intermediate can be used in anabolic reactions to synthetize amino acids, nitrogen bases, fatty acids, glucose

Metabolic pathways are regulated

Metabolic pathways are regulated at several levels, from within the cell and from outside by:

- the availability of substrate >regulation of metabolite concentration (production, uptake etc..)
- the activation or inhibition of the enzymatic activity >allosteric regulation; phosphorylation etc..
- regulating the amount of enzymes >regulation of gene expression and regulation of translation.

4-Chemical reactions in cells

Bioenergetics and thermodynamics

Definition of equilibrium equation, in which the concentration of our products is elevated to the stoichiometry coefficient, divided by the product of the concentration of reactants.

The tendency of a chemical reaction to go completion can be expressed as an **equilibrium constant**.

Usually, the constant of equilibrium is measured starting from standard condition (constant condition for each reaction and we start the reaction with the equimolar concentration of our compounds)

➤ The value of K_{eq} could be =1; >1;<1

-If the K_{eq} is equal to 1, this means that each compound is at the same concentration (the concentration compounds are equimolar).

-If the K_{eq} is higher than 1, this means that we have more products than reactants and this means that our reaction starts in standard condition and go versus the formation of the products.

-If the K_{eq} is lower than 1 this means that we have more reactant than products and in this case our equilibrium is shifted versus the formation of reactants.

➤ A large value of K_{eq} means that reaction tends to proceed until the reactants have been almost completely converted into the products.

In chemistry the constant of equilibrium is calculate at standard condition that are different from the condition in biochemistry:

Why? Because *in chemistry* we measure the constant equilibrium at the pressure of atmosphere and in the contrary *in biochemistry* the constant of equilibrium is calculated at condition where we have a pH equal to 7 (because is the physiological pH in cells)

In biochemistry, usually K_{eq} is calculated under standard conditions (298 K/25°C, pH=7, 1atm), the other standard conditions are the same, so products and reactants are initially present at 1M concentrations. At the end of the experiment, we can measure the real concentration of equilibrium and we can calculate the constant of equilibrium. In these conditions we defined the K'_{eq} . We use the notation K'_{eq} because it's indicated that we measured the constant of equilibrium at a condition with pH=7.

Standard free-energy change

Under the same standard conditions used for measuring K'_{eq} we can define the driving force that moves a system toward equilibrium: the standard free-energy change or Gibbs's standard free-energy

We know that when the standard free-energy change is negative: our reaction goes versus the formation of products and we have a release of energy, when the standard free-energy change is positive: the reaction goes versus the formation of reactants.

The relation that correlated the standard free-energy change and constant of equilibrium is

$$\Delta G'^{\circ} = -RT \ln K'_{eq}$$

→ variation of free-energy at standard condition, at pH=7 is equal to $-R$, T (temperature) and multiplied for the natural logarithm of constant of equilibrium. The value of free-energy change depends on the constant of equilibrium:

For a general reversible reaction:



Equilibrium equation:

Equilibrium constant

$$K_{eq} = \frac{[C]^c[D]^d}{[A]^a[B]^b}$$

Equilibrium constant expression

When the K_{eq} is:

TABLE 13-3 Relationships among K'_{eq} , ΔG° , and the Direction of Chemical Reactions under Standard Conditions

When K'_{eq} is ...	ΔG° is ...	Starting with all components at 1 M, the reaction ...
>1.0	negative	proceeds forward
1.0	zero	is at equilibrium
<1.0	positive	proceeds in reverse

>1, (log is positive) we have more products in equilibrium than reactants, the products is negative and we have an energy standard free-energy change.

=1, the standard free energy is equal to zero, change in our reaction, it's already in equilibrium and start in standard conditions

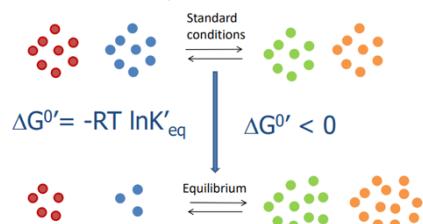
- <1, the logarithm is negative, so the product of the equation is positive, the standard free-energy is positive, and we have to remember that in this case the reaction is spontaneous versus the formation of reactants.

$\Delta G'0$ of a chemical reaction is simply an alternative mathematical way of expressing its equilibrium constant.

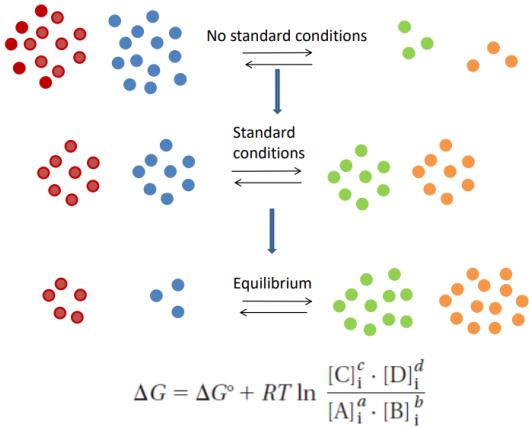
The standard free energy change $\Delta G'0$ tells us in which direction a reaction must go to reach equilibrium when the initial concentration of each component is 1.0M, the pH is 7.0, the temperature is 25°C, and the pressure is 1atm.

When the standard free energy is negative, it's spontaneous versus the formation of products: we have all the equimolar from the compounds (we start in a standard condition) and at the end of reaction we have more products than reactants:

If the variation of standard free energy is lower than 0, negative, it's spontaneous versus the formation of products



ACTUAL FREE-ENERGY CHANGE



In cells we don't have the same concentration in reactants and in products, so we cannot say if our reaction goes versus the formation of products or reactions (because we are not starting in a standard condition, but in a different condition).

- In the example on the right, we can see that we start from reactant concentrated more the products,
- During the reaction the compounds become at the same concentration, the reaction can go on to form products
- In this case we can measure the actual free energy change, that's more informative because in this way we can have an idea about what is the versus of our reaction.

This is an example of reaction that go versus the formation of products, but sometimes we can have the opposite.

The free-energy change can be calculated because it's related to standard condition: It's equal to the free energy standard condition + the value that depends on RT that are constant and the initial concentration of our reactant, we don't have the concentration at standard condition equimolar but the real concentration of the beginning of reaction. Cells can push some reaction versus the formation of products maintaining an high concentration of reactants.

This is a classic example: This reaction is the reaction in which 3-phosphoglycerate is moved to 2-phosphoglycerate. One of the reactions is a reversible reaction, so can go versus both formations, depending about if it's activated the glycolysis or not. At standard condition (that are not the condition in cells) the blood cells use a lot of glucose to produce energy, and the glycolysis is very activate and at the contrary red blood cells don't produce glucose. This reaction is a reaction where the equilibrium is shifted versus the formation of the reactants, so if we start at the same concentration

Example Calculation

Sample calculation of $\Delta G'0$ and ΔG .
 3-phosphoglycerate \rightleftharpoons 2-phosphoglycerate
 Given K_{eq} : $K_{eq} = \frac{2\text{-PG}}{3\text{-PG}} = 0.178$ at 37°C
 Calculate $\Delta G'0$

$$\Delta G'0 = -2.3 RT \ln 0.178$$

$$= -2.3(8.314)(310)(-0.75)$$

$$= +4446 \text{ J/mol or } 4.446 \text{ kJ/mol}$$

of **3-phosphoglycerate** and **2-phosphoglycerate**, we calculate the K_{eq} , that it's going to be positive, so it's lower than 1 and the change standard free energy results to be positive

Example Calculation

Calculate the overall free energy for this reaction, ΔG , using physiological conditions in a red cell (erythrocyte) at 37°C:

$$\begin{aligned} 3\text{-PG} &= 62.1 \mu\text{mol/liter} = 62.1 \times 10^{-6} \text{ M} \\ 2\text{-PG} &= 4.3 \mu\text{mol/liter} = 4.3 \times 10^{-6} \text{ M} \\ \Delta G &= \Delta G^{\circ} + 2.3 RT \log \frac{4.3 \times 10^{-6}}{62.1 \times 10^{-6}} \end{aligned}$$

In **red blood cells** the enzyme of reaction is regulated, and all the regulations allowed to maintain very high concentration of 3-phosphoglycerate and at the same time the concentration of 2-phosphoglycerate is low, because it's continuously transformed in another metabolism during glycolysis.

When glycolysis is activated the concentration of these two compounds (in red blood cells) are very different from standard condition, they are not equimolar.

Our reaction is pushed versus the formation of 2-

phosphoglycerate, because always maintain a low concentration.

In other cells like for example liver cells, the glycolysis go versus the degradation of glucose, but liver cells can also synthetized it by nucleogenesis, the activation/inhibition of different enzymes in our metabolic pathway can modified the concertation of 2/3-phosphoglycerate pushing the reaction versus the formation of 3-phosphoglycerate (in this way we can have the reverse reaction).

In case of endergonic reactions, ATP can hydrolyse(exergonic reaction) and free the energy needed for the reaction to occur
In cells we usually are not in standard condition so often when we see that in the reactions that we will see the situation is different.

The calculation of free energy gives us the idea if our reaction is **spontaneous or nonspontaneous**:

-spontaneous: release of free energy → exergonic reaction; The energy of reactants is higher than the energy of products, the difference between these 2 energies is the free energy change.

-nonspontaneous: absorb free energy → endergonic reaction. This energy can happen only if we give more energy to the system

Exergonic reactions ($\Delta G < 0$) are spontaneous
→ Release free energy

Endergonic reactions ($\Delta G > 0$) are nonspontaneous
→ Absorb free energy

$$\begin{aligned} \Delta G &= 4446 + 5928 \log 0.0692 \\ &= 4446 + 5928 (-1.16) \\ &= 4446 - 6876 \\ &= -2340 \text{ J/mol} \end{aligned}$$

The negative value of the overall ΔG for this reaction indicates that in the red cell it is spontaneous toward 2-PG.

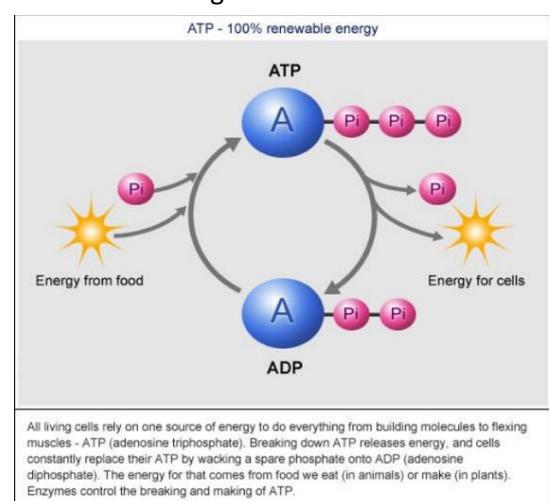
Adenosine triphosphate: the major carrier of chemical energy in all cells

Why we introduce the molecules of ATP? We know that the ATP is the most important carrier of energy in cells. A lot of organisms use ATP to give energy to endergonic reactions. The reaction is pushed versus the formation of products only if it's given energy, usually the molecules that do these things is ATP.

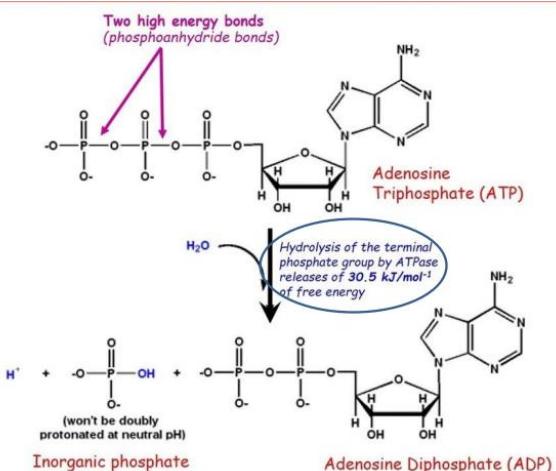
ATP> Is a nucleotide with triphosphate groups that are connected each other, forming very high energetic bonds. When these bonds are broken there is the release of energy because the hydrolysis of ATP into ADP, but also the hydrolysis of ADP into AMP release energy, because it's an exergonic reaction.

Usually, the energy is given by the hydrolysis of ADP into ADP, but sometimes is given by ADP in AMP.

From catabolism we have energy, the energy is concentrated in the molecules of ATP, and these molecules can use it to produce energy for anabolic reactions allowed the formation of molecules an macromolecules.



STRUCTURE OF ATP (non ricordare la formula chimica ma si la struttura)



-It's formed by nitrogen bases (adenine)

-By a sugar

-it's composed by different groups of inorganic phosphate. The energetic part of the molecules is at the level of the 3 phosphates because we have phosphoanhydride bonds (that release a lot of energy)

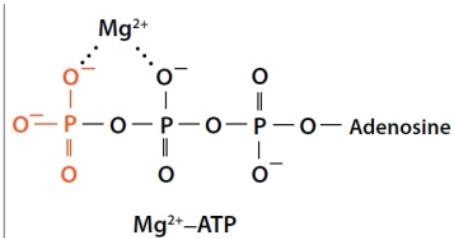
At the beginning the molecule of **ATP** is quite stable, but after the transformation in **ADP+phosphate**, is more stable, because in the ATP we have four negative charge very close to each other (there are different forces that try to break these bonds) and after the hydrolysis, the inorganic phosphate is very stable, and the 2 negative charge are divided.

ATP give energy during the hydrolysis cause later everything is more stable because there aren't repulsed forces. Products have lower energy than reactants. Exergonic reaction (release energy).

ΔG FOR ATP HYDROLYSIS IN INTACT CELLS

- The free-energy change for ATP hydrolysis is 30.5 kJ/mol under standard conditions, but the actual free energy of hydrolysis of ATP in living cells is very different: the cellular concentrations of ATP, ADP, and Pi are not identical and are far from standard conditions. (the free-energy change is higher than in standard conditions)
- ΔG for ATP hydrolysis in intact cells, usually designated ΔG_P ranges from -50 to -65 kJ/mol.
- Although the hydrolysis of ATP is highly exergonic, the molecule is kinetically stable at pH 7 because the activation energy for ATP hydrolysis is relatively high. Rapid cleavage of the phosphoanhydride bonds occurs only when catalyzed by an enzyme

The energy derived from ATP is used for coupled reactions. In coupled reactions, the energy from the hydrolysis of ATP is used to give energy to nonspontaneous reactions (endoergic)



ATP PROVIDES ENERGY BY GROUP TRANSFERS, NOT BY SIMPLE HYDROLYSIS

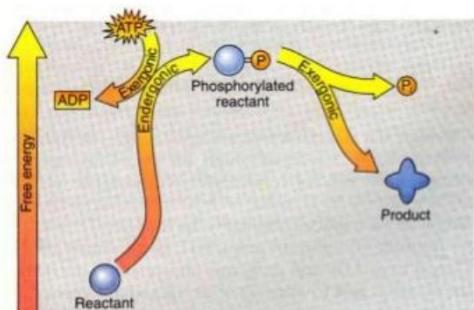


FIGURE 6-11 ATP transfers energy by transferring a phosphate group to a reactant. The reaction sequence is catalyzed by one or more specific enzymes. For simplicity, not all par-

When we have reaction in which energy of reactant is lower than the energy of products, we have a free-energy positive, so the reaction is nonspontaneous, and we have to add energy to push this reaction versus the formation of products.

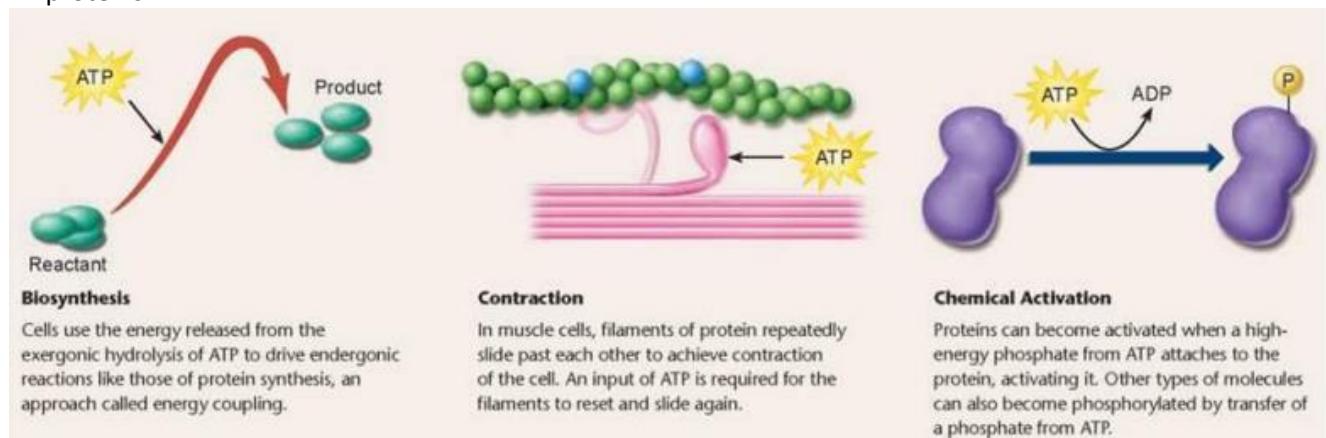
We have an intermediate reaction, in which ATP hydrolysed and the energy derived from this hydrolysed, is maintained inside to the enzyme to lift the reactant versus an intermediate, that is more energetic.

The phosphorylated reactant has an energy higher than products, so after the reaction can go versus the formation of products.

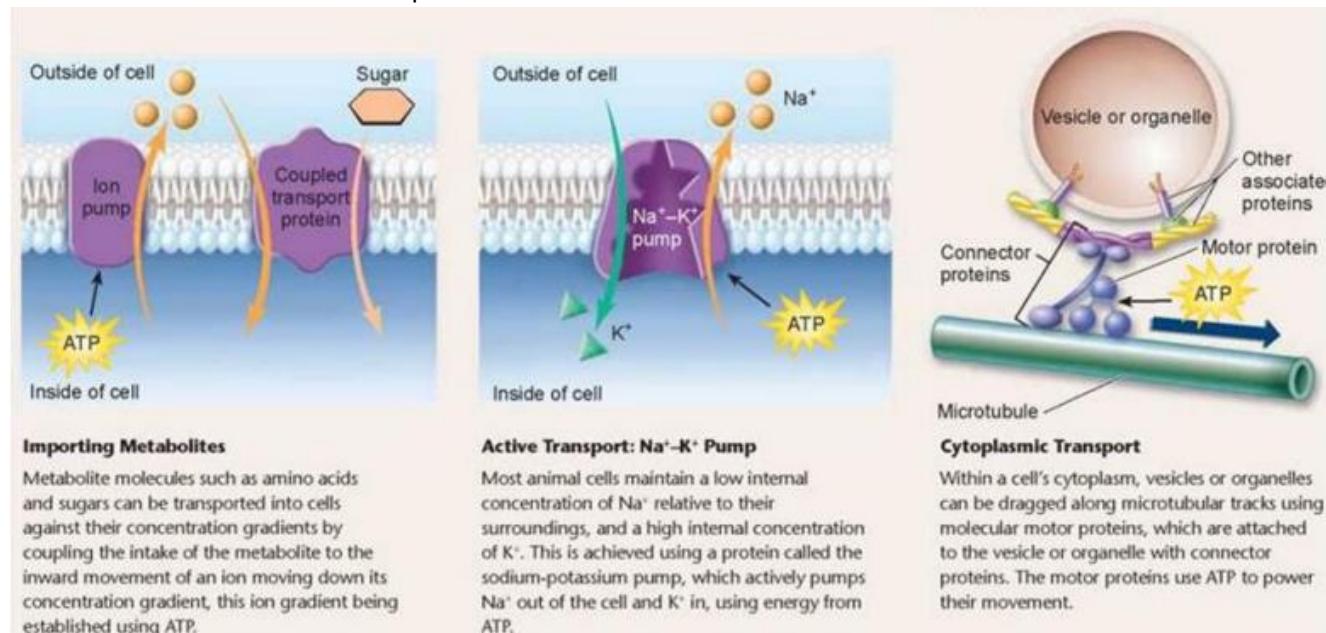
HOW DO CELLS USE ATP? The cells use ATP in different ways:

Reactants with ATP form an intermediate with a high energetic level than reactants and products, so in this way the reactant can be transformed in the products. ATP is used also for a lot of reactions and mechanism in cells, for example is used for allowing conformation changes in protein, an example of these conformation is important for the motility/ contraction of muscle. ATP can also activate proteins; the inorganic phosphate is transformed into proteins (protein phosphorylated); the presence of phosphate on the surface of the

protein can induce important conformational changes that can be important for the interaction between proteins.

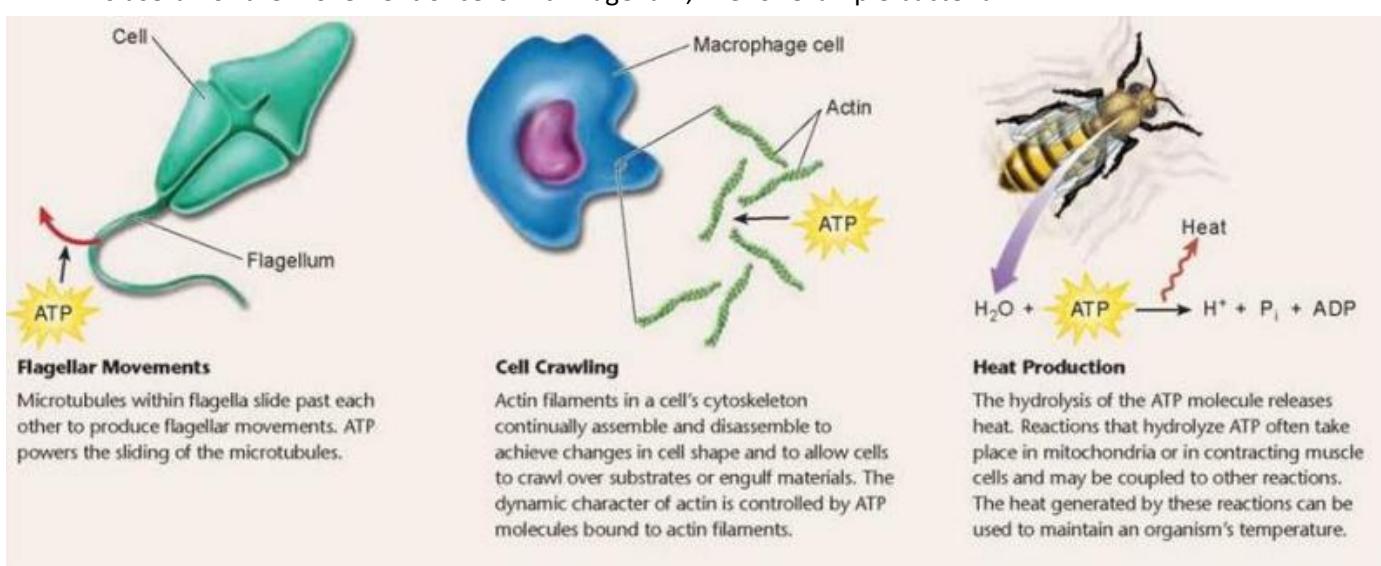


ATP is also useful for the transport of other molecules.



The energy is used for movement along microtubules to transport vesicle.

ATP is useful for the movement of cells with flagellum, like for example bacteria

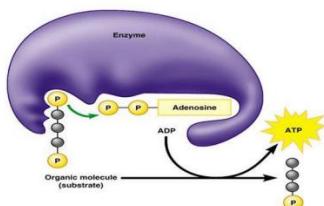


WHY IS ATP THE MAJOR CARRIER OF CHEMICAL ENERGY IN ALL CELLS?

ATP is the most important energetic molecule in cells. It can give energy for some reactions but can also take energy for other reactions; ATP can be recycled and can be continuously formed. If the ATP was the highest energy molecule, no other molecule could recharge it

THE TWO MAIN WAYS FOR PRODUCING ATP

* Substrate Level Phosphorylation

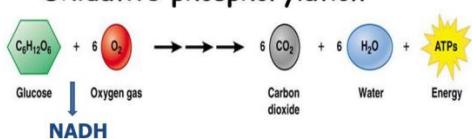


In cells we have 2 main mechanisms to produce ATP:

1) This mechanism required a molecule with a high energetic level of ATP, because from the hydrolysis of this higher energetic molecules energy is released and ADP can be phosphorylated to form ATP; these reactions are catalyzed by enzymes and this mechanism is called **SUBSTRATE LEVEL PHOSPHORYLATION** because we need a substrate to couple the exergonic reactions with reaction of phosphorylation of ATP (reverse reaction: exergonic reaction).

All cells can produce a high energetic molecule and can synthesize ATP starting from hydrolysis.

* Oxidative phosphorylation



2) **OXIDATIVE PHOSPHORYLATION** only in aerobic organisms (bacteria or cells, specific enzymes in bacteria) In this case the formation of ATP derives from the energy produced by the oxidative reaction during catabolism after glycolysis, in which the organic molecules are completely oxidized to carbon dioxide and water, the electrons with high energetic level derived from this catabolic reaction are used for reducing a proton gradient, that is useful for the synthesis of ATP by an enzyme that is called ATP SYNTHASE.

The difference is that in the substrate level we have a real substrate with a high energetic level and there is an hydrolysis reaction with synthesis of ATP ; in the second way to form ATP, it's formed exploiting a different concentration of protons, so we got a substrate and another form of energy. What makes the oxidative phosphorylation important is that the ATP is synthesized without a substrate.

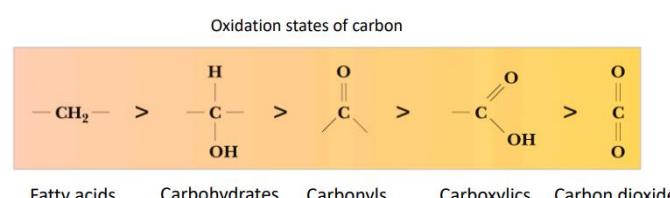
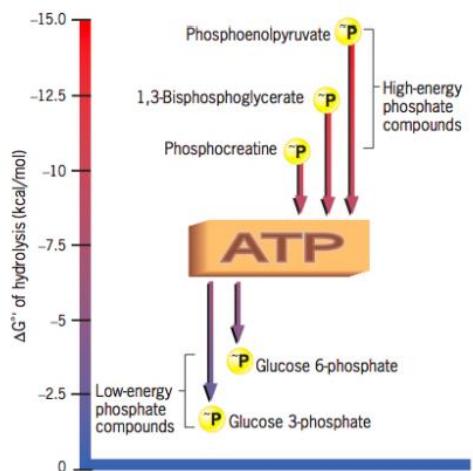
ROLE OF NAD+ AND NAD(P)H IN METABOLISM

The formation of ATP by oxidative phosphorylation derives from redox reactions that start from molecules that can transport electrons with high energetic levels: NADH (it's formed during redox reaction, that involves organic molecules and different levels of oxidation) and NADPH.

Catabolism is an oxidative process, in which, step by step, molecules switch from a reduced to an oxidized state giving electrons to NAD+ for producing NADH.

As we can see from the image we have molecules:

- that are very reduced/completely reduced: Fatty acids
- that are partially oxidized: Carbohydrates (we see that have an atom of oxygen; this means that this molecule is more oxidized than fatty acids)
- we have aldehydes and ketones, in which we have a carbon group, that is more oxidized than carbohydrates, in which we have hydroxyl group
- Here each carbon is in other status of oxidation



- Carbon dioxide: the more oxidized form of carbon

From fatty acids, carbohydrates, carbonyls and carboxylics, we have oxidation, so the electrons are extracted from these molecules and are transported by NAD (that is reduced to NADH), this energy is used in oxidative phosphorylation. In cells we can have the reverse reaction (in anabolic reaction) starting from carbon dioxide, for example photosynthesis cells can reduce more the organic molecules to form carboxylics , carbohydrates ecc. During the anabolism electrons are used for the reduce reactions (the molecules acquired energy and they form different organic molecules that can form macromolecules or storage molecules for energy. Anabolism is a process in which one passes from more oxidized molecules to more oxidized molecules. The electron donor is NADPH. When we see NAD usually is for catabolic reactions, but when we see NADPH or NAD+ we are talking about anabolic reactions

OXIDATION ENERGY OF MONOCARBONOUS COMPOUNDS

This slide explains us how energy is associated to different level of oxidation of organic molecules: Methane in this case is the most reduced form of carbon and from the table we can see that the oxidation of methane is higher than the other. Formic acid is an example of a molecule of carboxylic group, has a certain level of energy but is lower than the molecules that are more reduced. If a molecule is reduced give more energy than a molecule that it is in a different level of oxidation; we need to add energy to reduce molecules. The oxidation produces energy, the reduction require energy.

Photosynthesis produces a lot of ATP for reduction of carbon dioxide.

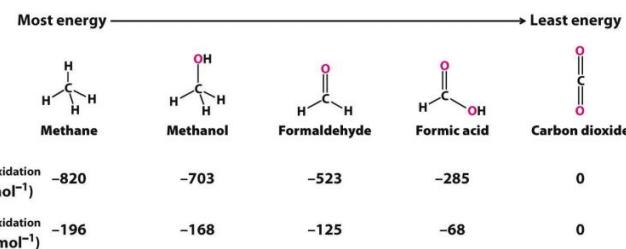
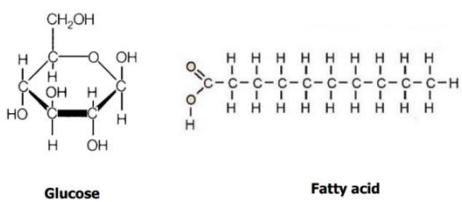


Figure 15.9
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ORGANIC MOLECULES AS FUEL FOR CELLS



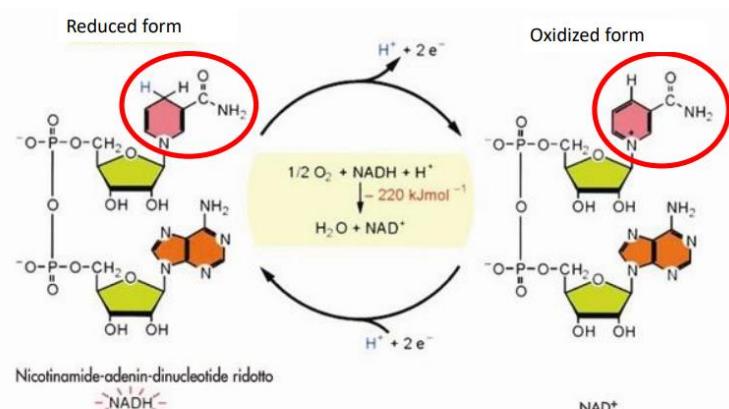
In cells the most important organic molecules are used for extracting energy for using ATP are glucose and general sugar, that can be transformed to form glucose, and also glucose is oxidized to produce energy or fatty acid. We see two molecules with high energetic, the high energetic level is completely used or partially reduced because we have hydroxylic group. How much energy we can extract from the oxidation of glucose and fatty acid?

OXIDATION ENERGY OF NADH/NAD⁺ COUPLE

This is the structure of NADH/NAD⁺ in the reduced or oxidate form; This is a very important molecule for the transport of electrons in cells.

The difference between NAD and NADP is a phosphate in the ribosome sugar, from a chemical point of view these molecules are equal but the presence of phosphate is important because some enzyme can interact NAD and some enzyme can bind NADP.

The presence of phosphate with negative charge it's important for the interaction with enzymes (because we have more negative charge compared with NAD). During the evolution anabolic enzymes are selected to interact with any NADP and catabolic enzymes are selected to interact with NAD, in this way cells know that if we have a lot of NAD in oxidate form, this means that we need energy because NADH is used for synthetized ATP and cells activate catabolism and all



the enzyme that interact with NAD can perform reaction and can synthetized NADH, on the contrary if we have a lot of NADH, enzyme for anabolism are activated for the anabolic pathway. **This is one of the ways that cells can regulate catabolism and anabolism, regulating the concentration of NAD or NADP.**

The characteristics of transport of electrons by these two molecules is in the circle red part of the molecule is nicotinamide group, that derives from a vitamin.

We have an adenine again nitrogen base, ribosome group and phosphate= this is a nucleotide.

The nitrogen base is different from the nitrogen that we find in the RNA or DNA, but the chemical characteristics are the same.

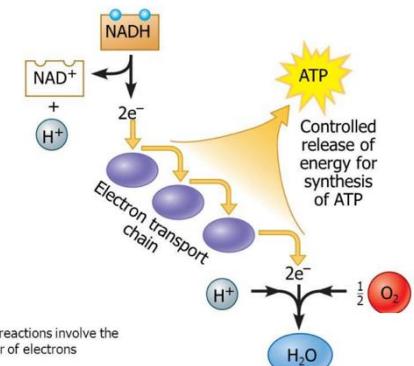
Nicotinamide-adenine has a specific characteristic: this group can react, can be reducer or oxidiser, double bond when is oxidate and this part of the enzyme can acquire two electrons and one proton to form the reduce part.

Remember: NAD or NADP can transport a couple of electrons (when they are reduced transport electrons, when they are oxidated release a couple of electrons).

The standard free energy of the reaction of oxidation of NADH into NAD+ is the reaction that is very exergonic at standard conditions (in fact -220 kJ/mol) and more exergonic in non-standard conditions (for example when we have a lot of ATP). NADH is a molecule with a high energetic potential.

NADH OXIDATION IS COUPLED TO ATP SYNTHESIS

During oxidation of NADH into mitochondrial or several aerobic bacteria, the couple of electrons in the NADH are transport across several enzyme that are called electrons transport chain (because chain of enzyme can transport electrons). We consume oxygen because of water, and the energy released from this redox reaction in the electrons transport chain is used for the synthesis of ATP. In this way the energy associated containing the NADH molecules is used to the synthesis of ATP, not directly.



$$\text{Energy Charge} = \frac{[ATP] + 0.5[ADP]}{[ATP] + [ADP] + [AMP]}$$

- Energy charge= $\frac{[ATP] + 0.5[ADP]}{[ATP] + [ADP] + [AMP]}$
- Indication of the energy status of the cell
 - Ability to perform work
- Energy charge at rest
 - Close to 1
 - Typically, 0.9-0.95
- Energy charge at complete exhaustion
 - Close to 0.75

ADENYLATE ENERGY CHARGE IN CELL

In this way cells can produce ATP and cells can maintained very high the concentration of ATP. We can measure the concentration of ADP and ATP. When we know all these concentrations we can calculate the energy charge of a cell, the energy condition of a cell. The energy charge of a cell is calculated considering the rate show of concentration of ATP and ADP, and the concentration of the total nucleotides of ADP, ATP and AMP. If our cell is in a good energetic level, we have a lot of ATP and we have a very low level of this nucleotides and so the energy charge of the cell is near to 1 (that is the maximum level of energy charge).

Cells need to maintain a high level of ATP because in this way cells have energy but in particular the free energy of the hydrolyse of ATP is very high, and the hydrolyse of the molecules of ATP give a lot of energy and can perform endergonic reactions, not spontaneous reactions.

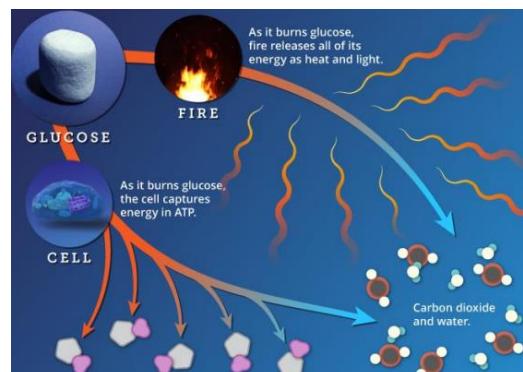
Usually, cells don't grow at energy charge level lower than 0.75, (still very high level of ATP), because need energy to maintain all the reactions require for life.

THE ENZYMES.

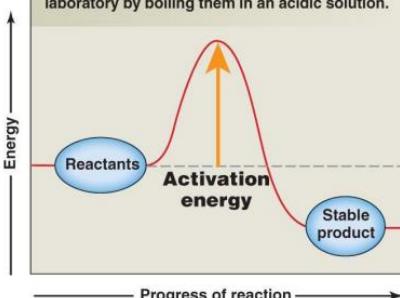
Virtually every chemical reaction in a cell occurs at a significant rate only because of the presence of enzymes, that catalyse them. Enzymes are catalytic molecules, that favour the equilibrium of reaction without interacting with the substrate. In cells the catalysis is performed by enzymes, that are in the most cases proteins (also RNA can catalyse some reactions), enzymes are very useful because they allow cells to perform reactions in very fast times, that are useful for the life of them. For example, if we want completely degraded a molecule of glucose at 25°C (that is the environment temperature) can be oxidized with the presence of oxygen, the reaction is exergonic reaction, so it is a spontaneous reaction; but we know that the standard free energy change give us information about the reaction that is spontaneous, but not give us information about velocity of our reaction.

At 25°C the complete oxidation occurs in about 100 years. At 180°C the complete oxidation occurs in about 5 minutes (this time is good for cells, but it's not good for the temperature that is very high). At 25°C in cells the complete oxidation occurs in few seconds. To activate this reaction we have to give to the molecules more kinetic energy for starting the collision/reaction.

ACTIVATION ENERGY



In the external environment, extreme conditions can provide the activation energy. For example, complex sugars can be broken down in a laboratory by boiling them in an acidic solution.



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Activation energy, in chemistry, the minimum amount of energy that is required to activate atoms or molecules to a condition (activate condition) in which they can undergo chemical transformation.

Why can enzymes catalyze this reaction and can increase the velocity of this reactions?

Because they act on the activation energy of the reaction. We have:

- reactants
- products, that are more stable than reactants.

Spontaneous reaction.

The velocity of the reaction depends on the activation energy.

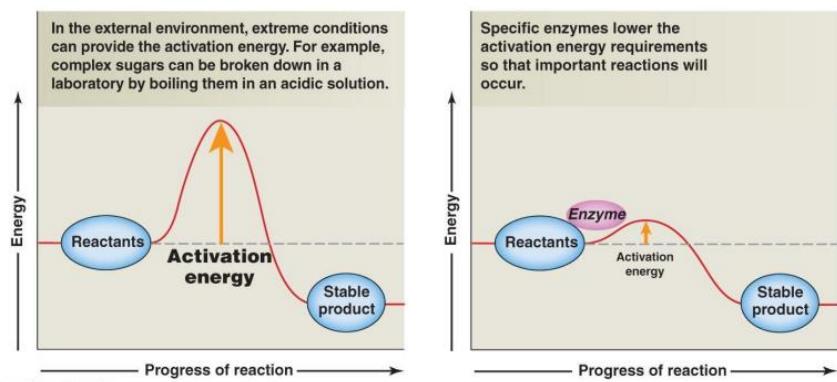
The higher the activation energy is, the slower is the reaction.

ENZYMES AFFECT REACTION RATES, NOT EQUILIBRIA

Enzymes are very important, because they interact with reactants and they can lower the activation energy, so more molecules are activated at enough energy to react and to form products, the important things that enzymes do is the reduction of activating energy.

Enzymes cannot modify the equilibrium of the reactants, if the reaction is spontaneous the free energy is the same, but the velocity of the enzymes can modify the velocity of our reactions. Decreasing the energy of activation, allowed a lot of molecules to do the reaction.

The action of protein catalysts called enzymes in promoting a chemical reaction by lowering its activation energy



ENZYMES ARE BIOLOGICAL CATALYSTS

- Enzymes increase the rate of the reaction from 10^5 to 10^{17} .
- Enzymes are very specific for their substrates, for each reaction in cells there are essential enzymes.
- Enzymes are very efficient.
- Enzymes are regulated.

TABLE 6-5 Some Rate Enhancements Produced by Enzymes

Cyclophilin	10^5
Carbonic anhydrase	10^7
Triose phosphate isomerase	10^9
Carboxypeptidase A	10^{11}
Phosphoglucomutase	10^{12}
Succinyl-CoA transferase	10^{13}
Urease	10^{14}
Orotidine monophosphate decarboxylase	10^{17}

5-Chemical reactions in cells

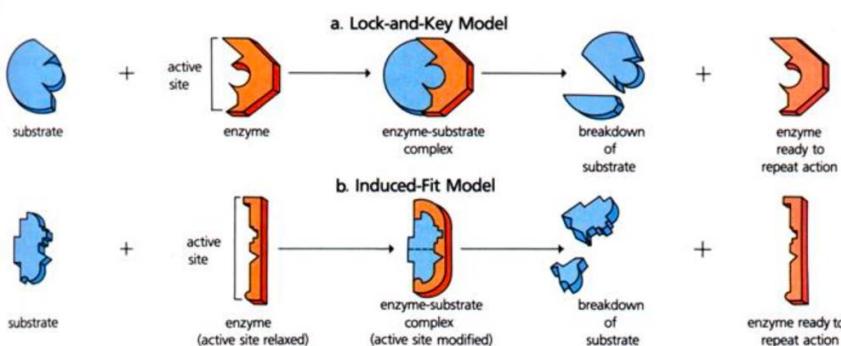
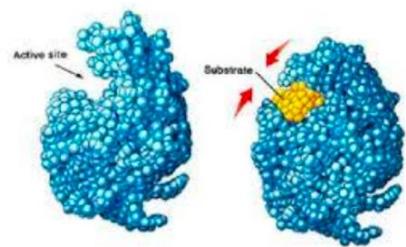
Enzymes are biological catalysts

In fact, the concentration of substrate is a method to regulate the activity of enzymes, if we have a lot of substrates the enzyme can perform the reaction very fast and if we have a lower level of substrate the reaction works in the opposite way.

- The function of enzymes is to lower the activation energy, enhancing the reaction rate.
- The equilibrium of a reaction is unaffected by the enzyme.
- Enzymes remain unchanged at the end of a chemical reaction
- Enzymes are, with a few exceptions, PROTEINS
- Substrate binding occurs in a pocket on the enzyme called the **active site**.

Enzymes can also have more than one active site

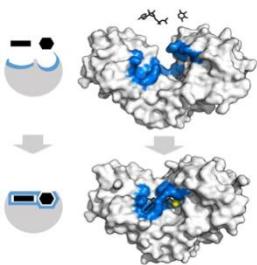
The active site is only a little part of the protein and only few amino acids can interact with the substrate. Some amino acids are important for the interaction with the substrate, in fact they bind the substrate and orient it in the right way for the reaction. Other amino acids are important for the real reaction: for activating the substrate and promoting several types of reaction. All the other amino acids are important for maintaining the structure of the enzyme and for its regulation.



Enzyme/substrate interaction models

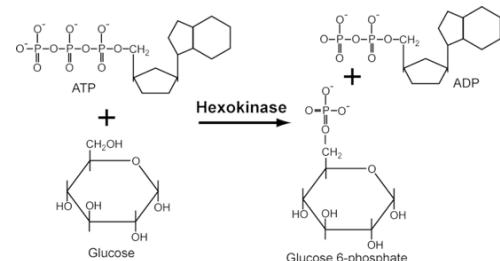
The first model that tried to explain the interaction between the enzyme and its substrate was called the **Lock-and-Key model**, in which it was supposed that the substrate can interact like a key inside a lock with the catalytic site. This model considered that the catalytic site was rigid, could not modify its structure.

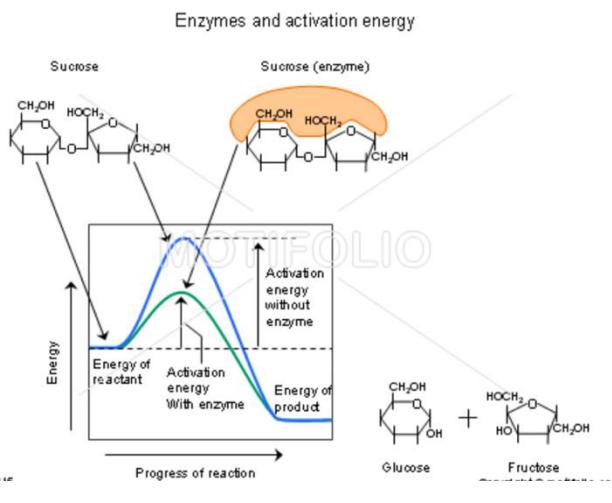
The best model that can explain the interaction is the Induced-Fit model, which considered that the active site has a structure that can interact with the substrate in a complementary way, but also when substrate interacts with some amino acids, the catalytic site can modify its structure and better fit the substrate, forming the enzyme-substrate complex. This is a dynamic model.



Conformational changes of hexokinase

This is an example of **conformational changes**: the enzyme hexokinase (catalyzes the first reaction of glycolysis) interacts with 2 substrates: ATP and glucose. During the reaction it is important that water is not inside the catalytic site: the enzyme can avoid it, in fact when the 2 substrates enter inside the catalytic site and interact with the amino acids, then the hexokinase changes its conformation in order to eliminate water from the catalytic site.



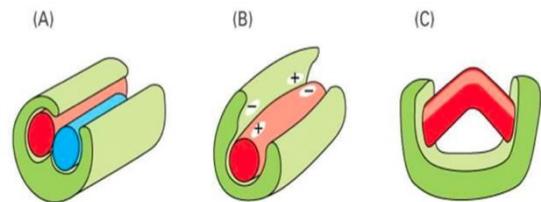


Enzymes and activation energy

Enzymes can reduce the activation energy of the reaction. For example, this enzyme can break a sucrose into a molecule of glucose and fructose.

When sucrose interacts with the catalytic site of the enzyme, some bonds are stretched and modified also adding partial charges). In this way the enzyme lowers the activation energy, because the new sucrose structure is more reactive than before

How do enzymes lower the activation energy?



enzyme binds to two substrate molecules and orients them precisely to encourage a reaction to occur between them

binding of substrate to enzyme rearranges electrons in the substrate, creating partial negative and positive charges that favor a reaction

enzyme strains the bound substrate molecule, forcing it toward a transition state to favor a reaction

Cofactors and coenzymes

A cofactor is a non-protein chemical compound or metallic ion that is required for an enzyme's activity.

Cofactors can be considered "helper molecules" that assist in biochemical transformations.

We can classify cofactors in 2 big groups:

- inorganic ions
- complex organic molecules, which are also called coenzymes

Coenzymes= organic cofactors, they can be sub-classified in 2 groups:

- some coenzymes can exit and enter the catalytic site
- some coenzymes are tightly bound to the catalytic site (or covalently bound) >in this case called prosthetic group

A lot of coenzymes derive from vitamins, we need small amount of coenzymes, and so of vitamins, but they are essential for our life because we cannot synthesize vitamins (humans). Depending on the vitamin and so on the coenzyme that is formed, each coenzyme usually is involved in a specific group of reactions.

TABLE 6-2 Some Coenzymes That Serve as Transient Carriers of Specific Atoms or Functional Groups		
Coenzyme	Examples of chemical groups transferred	Dietary precursor in mammals
Biocytin	CO ₂	Biotin
Coenzyme A	Acyl groups	Pantothenic acid and other compounds
5'-Deoxyadenosylcobalamin (coenzyme B ₁₂)	H atoms and alkyl groups	Vitamin B ₁₂
Flavin adenine dinucleotide	Electrons	Riboflavin (vitamin B ₂)
Lipoate	Electrons and acyl groups	Not required in diet
Nicotinamide adenine dinucleotide	Hydride ion (:H ⁻)	Nicotinic acid (niacin)
Pyridoxal phosphate	Amino groups	Pyridoxine (vitamin B ₆)
Tetrahydrofolate	One-carbon groups	Folate
Thiamine pyrophosphate	Aldehydes	Thiamine (vitamin B ₁)

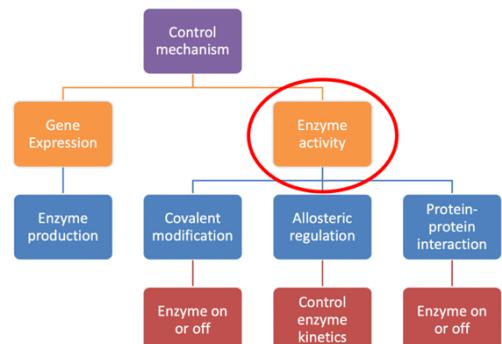
Enzymes regulation

We have several control mechanisms for the regulation of enzymes.

Cells can regulate the activity of the enzyme regulating the expression of the protein, so in cells there are some mechanisms where transcription factors are activated, inducing gene expression for specific enzymes that are required. This is a long-term regulation

On the contrary, when we have the enzymes inside the cell they can be regulated in different ways:

- 1) Regulation of the concentration of the substrate
- 2) Regulation by inhibition/activation > both at the level of the substrate but also by other mechanisms which are:
 - covalent modification of the enzyme (like phosphorylation)
 - allosteric regulation (increasing or decreasing kinetics parameters of enzymes)
 - protein-protein interaction (some protein can interact with the enzyme and activate or inhibit it)



Substrate concentration affects the rate of enzyme-catalyzed reactions

Kinetic parameters are important when we are studying an enzymatic activity.

We start considering the reaction: in this case we have a simple enzyme that can interact only with one substrate (S)

$SE \rightleftharpoons E + P$ complex enzyme-substrate > only when this complex is formed the substrate can be transformed into the product

If we analyze the reaction at the beginning (when we have only substrate and no products), we can consider that the rate of our reaction depends on the kinetics of the formation of the substrate-enzyme complex and the kinetic constant of the transformation of the substrate into the product.

So, in the 2 arrows of the reaction we have, in each arrow, a kinetic constant.

Usually, the kinetics characteristics of the enzyme are studied in these particular conditions (when we have only the substrate and so the reaction is pushed versus the formation of the product).

If we measure during time the concentration of the products that are formed, we can then measure for each increasing concentration of substrate the initial velocity of the reaction. And, adding more and more concentration of substrate, our reaction goes faster until a plateau.

In fact if you replot the data in a second graph, in which you correlate the concentrations of substrate and the initial velocity of the reaction, you can see that at low amount of substrate the initial velocity increases in a linear way, but if you add a lot of substrate at the end you reach a plateau. Then when the velocity doesn't change anymore adding a lot of substrate you reach the maximum velocity of the enzyme.

(in this experiments the concentrations of the enzyme are maintained fixed, what changes are the concentrations of the substrate)

In conclusion, all the enzymes that interact with a substrate follow a hyperbolic behavior when adding different concentrations of substrate.

We can explain the hyperbolic behavior, in fact, we can see that at low concentrations of substrate some enzymes can interact with the substrate (forming the product), while other enzymes cannot interact with the substrate (because we have few molecules of substrate) and so we have low reaction rate.

Increasing the substrate, more enzymes can interact with the substrate and so we can see that the reaction rate increases.

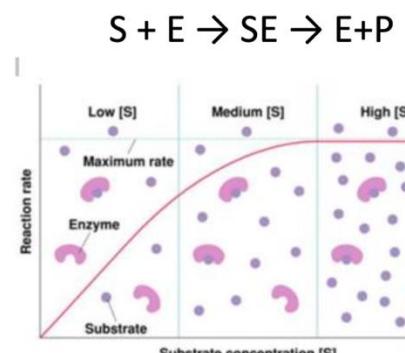
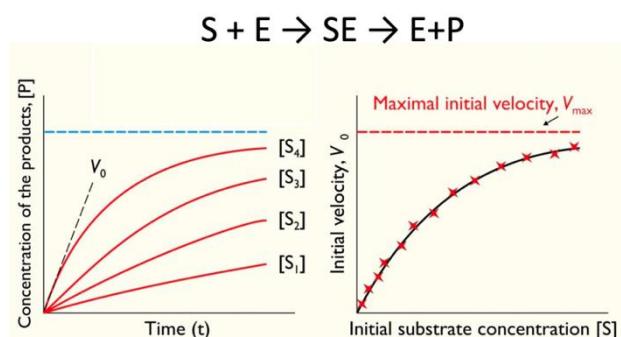
This continues until we add too much substrate and so we have higher concentration of substrates with respect to the concentration of enzymes > all the catalytic sites of our enzymes are occupied by a substrate, so the velocity is at its maximum.

At the end of the 19th century, Michaelis and Menten, described this behavior of enzymes with an equation, which correlates the concentration of substrate with the initial rate of the reaction.

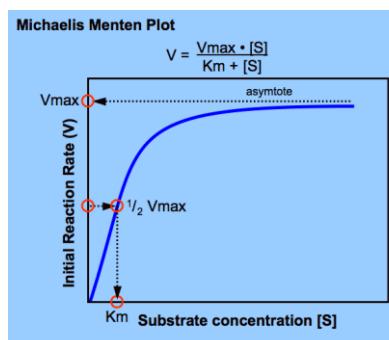
In the equation the 2 variables are connected by 2 constants, which are the most important kinetic constants of enzymes:

- V_{max} > represents the maximum rate achieved by the system which happens at saturating substrate concentration.

- K_m > called Michaelis-Menten constant, it is numerically equal to the substrate concentration at which the reaction rate is half of V_{max} . Inside the K_m , all the kinetic constants of the reaction are considered. K_m gives us an idea on what is the affinity of the enzyme with its substrate. If the K_m is very low, it means that the formation of the enzyme complex is very quick and so it means that the substrate has high affinity with the enzyme, on the contrary when the K_m is high, the substrate has lower affinity with its enzyme.

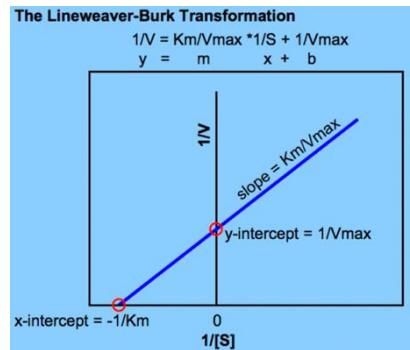


The kinetic curve has an hyperbolic shape



The important terms are [S], V, V_{max}, and a constant called the Michaelis-Menten constant, K_m. All these terms are readily measured experimentally.

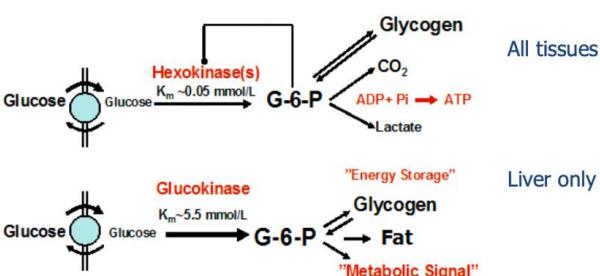
This is another way to represent an enzymatic activity:
Lineweaver and Burk transformed mathematically the Michaelis and Menten equation, obtaining the linearization of the equation.
They did it by using the reciprocal of V and of S.
So with this transformation it is easier to identify the terms.



Kinetic parameters are used to compare enzyme activities

This is an example of how it is important to know the kinetic parameters of enzymes.

In our body we have different isoforms(isoforms are enzymes that catalyze the same reaction but have different kinetic parameters) of hexokinase(starts glycolysis).



All our tissues, except the liver, express the classical isoform of hexokinase, called hexokinase (s), which has a K_m equal to 0.05 mmol/L. In liver we have an isoform of the same enzyme, called glucokinase, which has a K_m 100 times higher (5.5) than the hexokinase in other tissues.

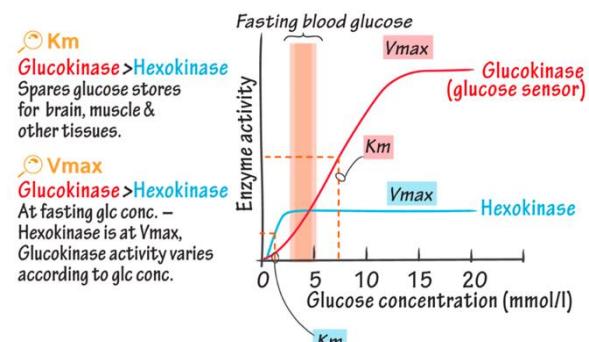
This is important for our physiology because liver is the master regulator of glycemia (=concentration of glucose in our blood) in our body. Liver usually produces glucose for the other organs,

possibly without using glucose for its own energy. So glucose doesn't use glucose for its glycolysis > this is why in liver we have an enzyme with higher K_m : because in this way liver can use the glucose only when we have an high concentration of glucose.

In our blood the concentration of glucose is maintained constant: in resting conditions it is around 5 mmol/L. This means inside the cells of all the tissues the hexokinase is working at its maximum velocity, so when glucose enters the cells, it is immediately phosphorylated, and it can enter in glycolysis or in another pathways.

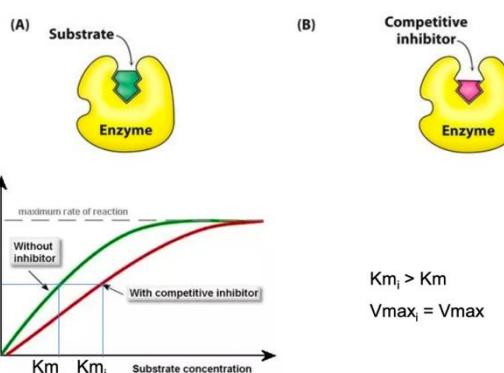
On the contrary, liver can intake glucose, but glucose is not phosphorylated (or it is phosphorylated very slowly) > in this way when we have normal concentrations (or lower when fasting) of 5 mmol/L, then liver uses glucose very slowly.

When the concentrations of glucose increase in our blood (for example after a meal), the other tissues can use glucose in the same way as before, but also the glucokinase increases its activity > in this case also glucose in liver is phosphorylated and liver can use glucose.



Regulation by controlling enzyme kinetics: competitive inhibition

This is one of the mechanisms of inhibition:



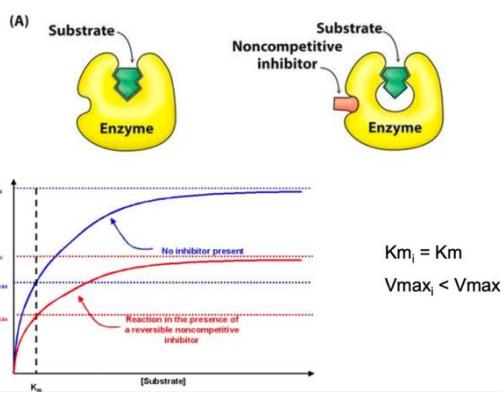
A **competitive inhibitor** is a molecule that resembles the normal substrate, binds to the enzyme and prevents the substrate from binding.

This inhibitor competes for the catalytic site with the substrate. The result of this is that the initial rate of the reaction is lower than in absence of the inhibitor, also you can still reach the V_{max} but with a higher amount of substrate.

We have to consider that the interaction inhibitor-enzyme is an equilibrium interaction (like the substrate-enzyme), so the inhibitors can exit from the catalytic site in a dynamical

equilibrium > so if you add a lot of substrate, then the substrate can overcome the inhibition. The K_m in presence of the inhibitor is higher than in absence of the inhibitor, so in appearance the substrate has less affinity for the enzyme compared to non-inhibited conditions. This is because we need more substrate to reach the V_{max} . In these cases we talk about apparent K_m (because it is not the real K_m , but it is modified by the presence of the inhibitor).

Regulation by controlling enzyme kinetics: non-competitive inhibition



We can have some metabolites that do not have a structure similar to the substrate, so they cannot enter inside the catalytic site. But they can interact with another part of the protein, inducing conformational changes in the protein that modify the structure of the catalytic site.

A **non-competitive inhibitor** is a molecule that binds the enzyme in a site different to active site hampering substrate transformation. When a non-competitive inhibitor interacts with the enzyme the substrate cannot enter, or can enter but cannot be modified, in the catalytic site.

The result is that in presence of the inhibitor we have a reduced V_{max} and, in this case, even if we add more and more substrate, the real V_{max} is not reached > because the enzyme that binds the non-competitive inhibitor is always inhibited, the substrate cannot compete with the inhibitor.

The V_{max} are different, but the shape of our hyperbole is the same, so when you calculate the K_m it will be the same also in presence of the inhibitor.

Regulation by controlling enzyme kinetics un-competitive inhibition

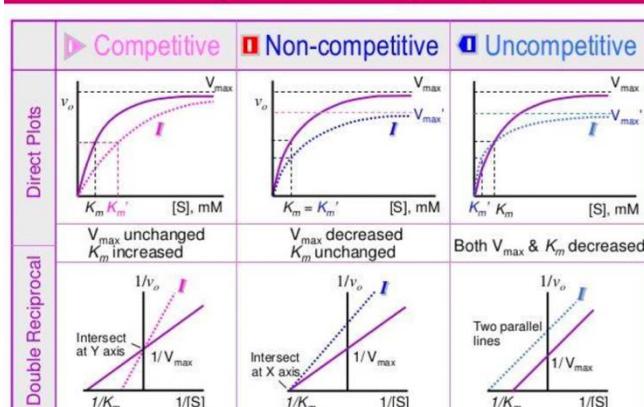
An **un-competitive inhibitor** is a molecule that binds the enzyme near to the active site after the binding of substrate and blocks the substrate in the catalytic site.

When the catalytic site of the enzyme is empty, the un-competitive inhibitor cannot interact with the enzyme.

In this case both the kinetic parameters change: in presence of the inhibitor we have a reduced V_{max} , but we also have a reduced K_m .

In this case we cannot say that the substrate has higher or lower affinity for the enzyme.

Enzyme Inhibition (Plots)

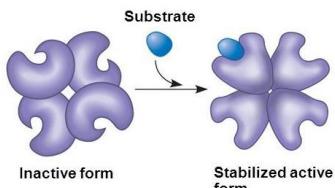


The reason why the K_m is lower in presence of the inhibitor is that when the inhibitor binds the enzyme the substrate cannot exit anymore.

Regulation by controlling enzyme kinetics

An easy way to study the kinetics of inhibition is to replot the direct plots of inhibition with also the Lineweaver-Burk representations. In this way is very easy to understand the type of inhibitor.

Allosteric regulation



Allosteric regulation is a specific regulation for some enzymes, that have different characteristics compared to the enzymes that followed the Michaelis and Menten kinetics. In fact, now we are talking about enzymes that are formed by more than one subunit and so they have more than one catalytic site. The simplest allosteric enzymes are enzymes formed by 2/3/4 identical subunits (each with a catalytic site). So, the

subunits catalyze the same reaction, but the subunits can also interact with each other, modifying the kinetic characteristics of their catalytic sites.

Allosteric enzymes are polypeptides.

Each enzyme has active and inactive forms > this means that, in inactive form, all the catalytic sites are in a structure that does not have an high affinity for the substrate, so the substrate has some difficulties in entering the catalytic site. The active form, on the contrary, is a form in which we have all the catalytic sites open and organized in a structure that can interact easily with the substrate.

Each polypeptide can amplify the enzymatic activity of neighboring enzyme:

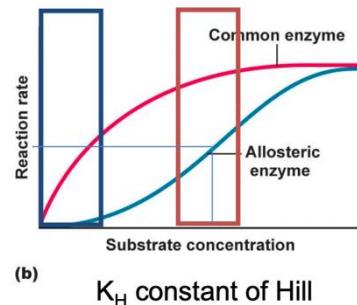
The interaction of a molecule of substrate with one of the subunits can induce a conformation change to the catalytic site of the subunit, and this activates the catalytic site of the subunit but also, in a cooperative manner, there are conformational changes of the other subunits, activating their catalytic sites.

The kinetic curve has a sigmoidal shape

A lot of regulated enzymes are allosteric enzymes, because in this way at the beginning of the reaction they are not very responsive to the substrate, but then, at certain concentrations (near to the flexus of the sigmoid) these enzymes can modify very quickly the reaction rate for a little variation of substrate concentration.

This is important, because during metabolism, some metabolites can have little changes in their concentrations, to which allosteric enzymes are sensitive and so they can pass from an activity with low reaction rate to an activity with high reaction rate for little variation of substrate.

Also in this case, the concentration of substrate corresponding to half of the V_{max} , is a constant called constant of Hill, which is useful because its value gives us the number of catalytic sites in our enzyme.

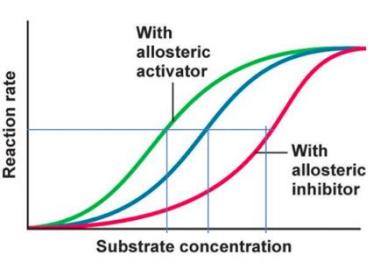
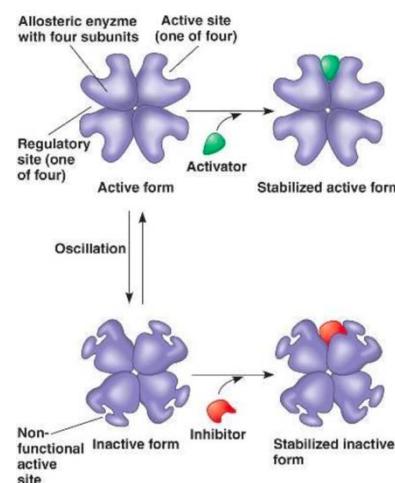


Also allosteric enzymes can be regulated by inhibitors and activators.

In this case the inhibitor/activator acts in a part of the enzyme that is not the catalytic site, but it is often a part between different subunits.

Allosteric enzymes are present in an equilibrium between the active and inactive form:

- The binding of an activator stabilizes the active form, also in absence of the substrate. Because the activator shifts the equilibrium versus the active form. Now the enzyme can interact directly with the substrate.
- The binding of an inhibitor stabilizes the inactive form, so the equilibrium is shifted versus the formation of the inactive form. This means that we need more and more substrate to activate the enzyme.

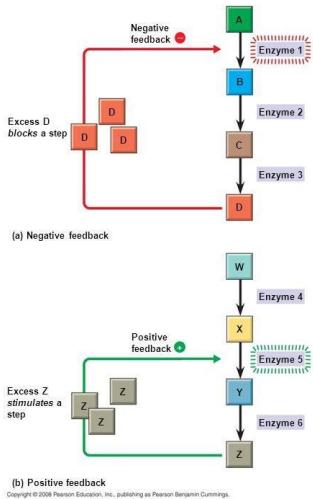


(Blue line describes the enzyme in absence of activator/inhibitor)

From the graph we can see that in presence of an activator, the V_{max} reached is the same, but the enzyme increases the reaction rate at lower concentrations of substrate.

On the contrary, when we have an inhibitor, we need to add more substrate to activate the catalytic sites.

The only kinetic parameter that changes is the K_H , that is lower in allosteric activator and higher in allosteric inhibitor.



- Negative feedback: one of the final products inhibits the first enzyme of the metabolic pathway. This means that, when we have an accumulation of our products, they can act as inhibitors to allosteric enzymes. Whereas, if we have a Michaelis-Menten enzyme, the product acts as a non-competitive inhibitor

- Positive feedback: one of the products activates the metabolic pathway. The product can stabilize the active form of an allosteric enzyme, pushing the reaction forward.

6-Enzymology and glycolysis

Another mechanism for the regulation of enzymes is the **protein-protein interactions**.

We can have some proteins that are inhibited or activated by interactions with other proteins.

This is a typical example:

Kinase A (a kinase is an enzyme that can phosphorylate something) can phosphorylate proteins during signal transduction. Protein kinase in cells is a soluble enzyme that is formed, in its inactive form, by two catalytic subunits that can interact with two regulatory subunits. These two subunits inhibit the catalytic subunits and when the cell are in arresting state, PKA (cAMP dependent protein kinase A) is not active.

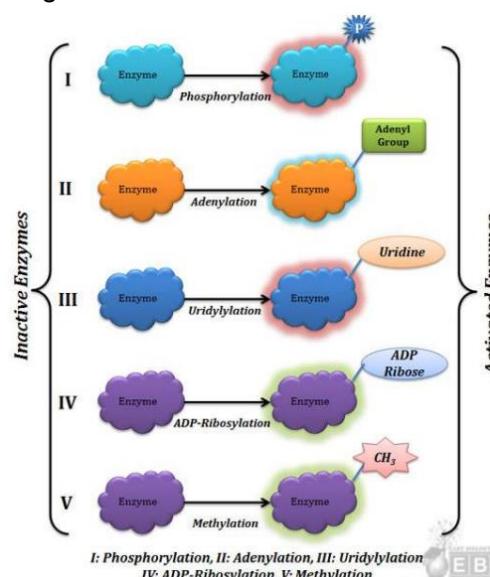
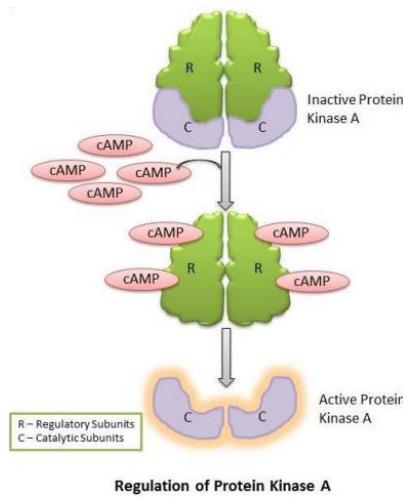
However, a signal, created after the activation of a receptor in cell (usually protein kinase is activated by hormones like glucagone), induces the formation of cAMP (cyclin AMP; allosteric regulator; derives from ATP). This molecule is present at a low level in cells at resting conditions but we can increase its concentration during signal transduction.

When cAMP increase in cytosol, it can interact with regulatory subunits, which change their conformation. Now, they are not anymore specific for their catalytic subunits, which are released in cytosol. At this point, the catalytic subunits are free and the PKA can interact with its substrates and phosphorylate several protein, which are often enzymes for glucose metabolism

Another mechanism for the regulation of enzymes is the **covalent modification**, for example, phosphorylation. Cells can phosphorylate an enzyme, and phosphorylation induces conformational change because we add a P group, that has 2 negative charges. These 2 neg charges can induce a conformational change because they can interact for example with some positive aminoacid or on the contrary negative amino acid are repulsed by the phosphate. So the change of the conformation can activate it or inhibit it.

The same occurs after others modification such as the addition of an adenyl group, a uridine or ADP ribose. In all these covalent modification nucleotides are involved (ATP, UMP, CTP).

Another case is the methylation (+CH₃) of proteins, for ex of an histone.



Covalent modification is necessary to have enzymes that can break the covalent bonds to modify the enzyme not in its activated or inactivated form (ex phosphorylated enzymes we have phosphates). For each enzyme that can induce a covalent modification, we have the opposite enzyme that does

opposite reaction. In this way, we'll have always a balance between the activated and inactivated enzyme depending on the metabolic condition.

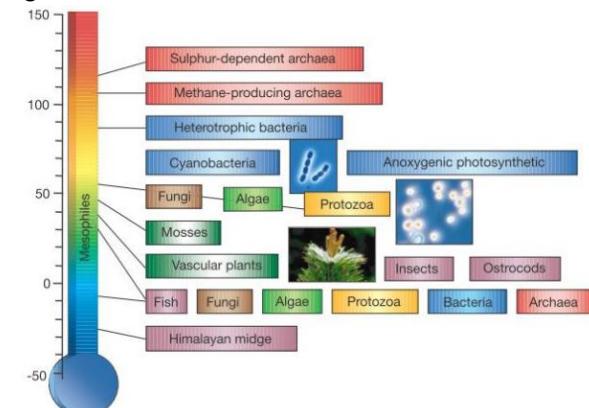
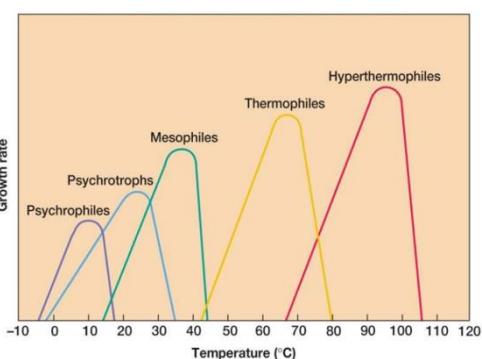
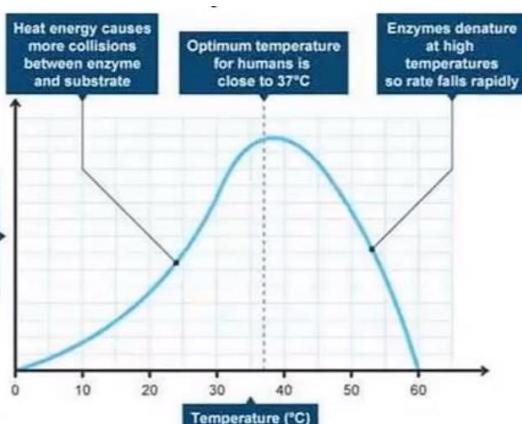
We have to consider that the enzymes works at a specific temperature called **optimum temperature**.

Each enzyme has a specific optimal temperature: for homothermal animals (also humans) is around 37°. You see that lower the temperature, lower is the enzymatic activity (less collisions, lower kinetical energy). But also at higher temperature the rate of reaction decreases because the stability of the proteins decrease and at the end the protein is denatured.

In nature we have different organism that can live at different temperatures.

Extremophilic organisms can live at very low temperature (around -10°). They have protein that at that temperature are stable and in their bloods they have a protein that works as antifreezing liquid.

Extremophilic organism : Taq polymerase of thermophilic bacteria is very stable at very high temperature (PCR).



GLYCOLYSIS

Now we can start with metabolism and in particular with the most important metabolic pathway which is **glycolysis**.

The carbon hydrated metabolism is central in metabolism because from glucose and its intermediates we can synthetize amino acids, lipids, ecc.

Glucose has a central position in the metabolism of every organism because it is a very versatile molecule. It can also give a lot of energy after its complete oxidation.

Oxidation 1 mol of glucose = 180 g of glucose

After the complete oxidation of 1 mol of glucose to carbon dioxide and water, the standard free energy obtained is of -2840 kJ/mol.

Glucose is important in our metabolism because it can form many intermediates for different metabolic pathway.

Glycolysis literally mean "splitting", the degradation of glucose. All the living organisms can perform glycolysis. Some cells can only performed it to produce energy (ex blood cells).

It occurs in cytosol.

Four Major Pathways of Glucose Utilization

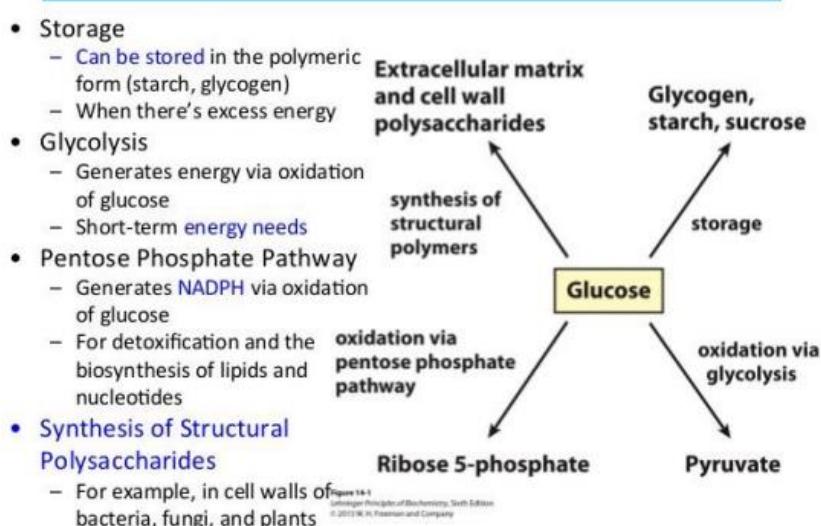


Figure 18-1
Lodish et al., Molecular Cell Biology, Sixth Edition
© 2013 W.H. Freeman and Company

During the degradation of glucose we have several steps (too much energy released for only one reaction). Step by step the energy of the oxidation is extract and maintained in the two most important molecules in catabolism: NADH, which can transport electrons at high energetic level, and ATP, the energetic molecule that can directly be used for other reactions in which energy is required.

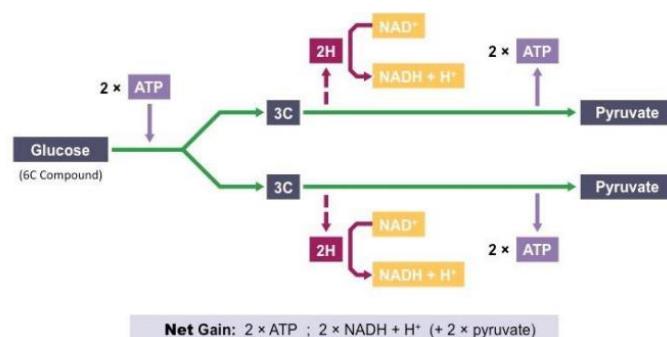
The glycolysis can be divided in two phases (from a teaching point of view):

- Preparatory phase (1-5 steps): molecules are modified and prepared to be more reactive and to produce energy. We do not have energy production but the opposite, energy consumption.

Glucose (6 carbon) using 2 molecules of ATP (energy consumption) is split and two molecules with 3C are produced.

- Payoff phase (5-10 steps): we have energy production.

Each of 3C molecule is oxidized to form pyruvate (the last product). During this phase we have an oxidation with the formation of NADH for each compound of 3C ($\rightarrow 2 \text{ NADH}$). And after we have the formation of 1 molecule of ATP for each reaction ($\rightarrow 2 \text{ ATP}$).

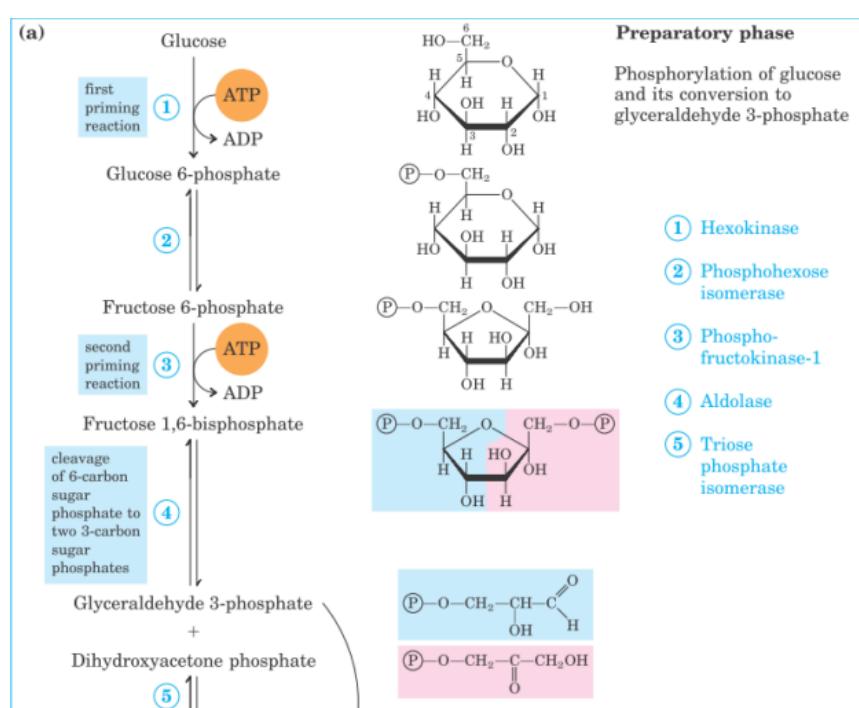


The most important thing to remember is that the 1 molecule of glucose is oxidized in two molecules of pyruvate with the formation of 2 NADH and 2 ATP.

PREPARATORY PHASE

We have 5 reactions:

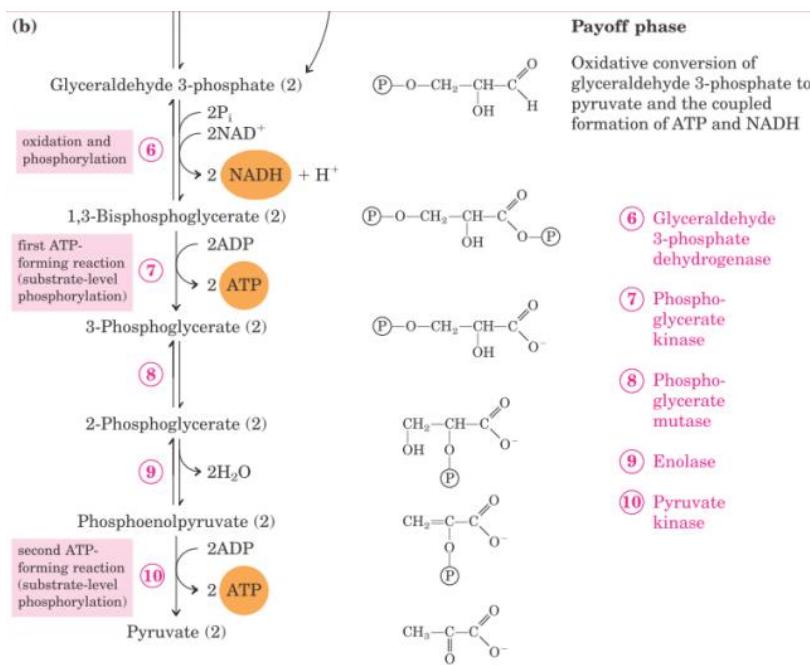
1. Glucose is phosphorylated to form glucose-6-phosphate. [consumption ATP]
2. glucose-6-phosphate is isomerized into fructose-6-phosphate. An isomer is a molecule with the same molecular formula but with a different structure. In this case we pass from an hexane to a pentane.
3. fructose-6-phosphate is phosphorylated to fructose-1,6-phosphate. [consumption ATP]
4. fructose-1,6-phosphate. This molecule is very unstable and it can be split in two molecules with 3 carbons that are glyceraldehyde 3-phosphate and the dihydroxyacetone phosphate
5. dihydroxyacetone phosphate is isomerized in a molecule of glyceraldehyde 3-phosphate. So at the end, we have 2 molecules of glyceraldehyde 3-phosphate.



PAYOUT PHASE (here we have the consumption of 2 molecules of ATP)

6. glyceraldehyde 3-phosphate (2) are oxidized to form a molecule with a high energetic level which is called 1,3-biphosphoglycerate (2).
7. 1,3-biphosphoglycerate (2) are phosphorylated to form 3-phosphoglycerate (2), gives the energy (hydrolysis of phosphate is used for the synthesis of ATP).
8. 3-phosphoglycerate (2) is modified into 2-phosphoglycerate (2).
9. 2-phosphoglycerate (2) transformed in phosphoenolpyruvate(2).
10. Phosphoenolpyruvate (2) become pyruvate(2).

Both reaction 7 and 10 are reaction in which ATP is synthetised at substrate level since oxygen is not involved.



STEP 1: Phosphorylation of glucose to glucose-6-phosphate.

It is an irreversible reaction because it is coupled with hydrolysis of ATP. Phosphorylation is an endergonic reaction but if we couple it with the hydrolysis of ATP, it becomes that the general energetic balance has a negative free standard energy and so it is an exergonic reaction.

The phosphorylation of glucose to glucose-6-phosphate is important for two reasons:

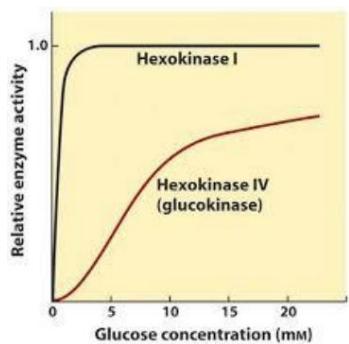
1. Has a higher energetic level. In this way it can undergo to the other reactions, which, most of them, are exergonic too.
2. After the phosphorylation, glucose acquires 2 negative charges (phosphate group) and cannot diffuse across the membranes. It is a hydrophilic molecule (it is brought inside the membrane by carriers) so it should not diffuse outside but it can happen if it is present at high concentration. After phosphorylation it is impossible because of the negative charges.

Phosphorylation is necessary to retain phosphorylated intermediates in the cell.

The enzyme that catalyses this reaction is the **hexokinase**. Thanks to it, ATP is not hydrolysed by water, instead after its hydrolysis the phosphate can interact with hydroxyl groups of glucose.

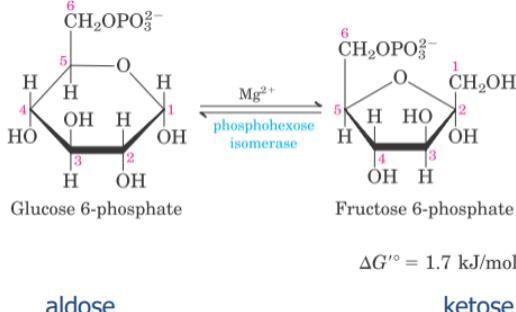
Important to know the K_m of enzymes because in all the tissues we have an isoform of hexokinase where $K_m = 0.05 \text{ mM}$ and in liver we have an isoform which is called glucokinase, whose $K_m = 5 \text{ mM}$.

Very important because in normal condition we have a glucose concentration in blood of 5-7 mM and all hexokinase in all the tissues are working at the maximum velocity. On the contrary, the hexokinase in liver is working around half of its maximum velocity. Important because liver spares sugar for the other tissues and liver can use sugar in an efficient way only when we have an increase of glucose in blood. In this case, liver can update glucose and phosphorylate it using hexokinase. Phosphorylation is efficient only when glucose is at higher concentration than physiological concentration (5-7 mM).



STEP 2: Conversion of glucose-6-phosphate to fructose-6-phosphate.

Enzyme=**phosphohexose isomerase**

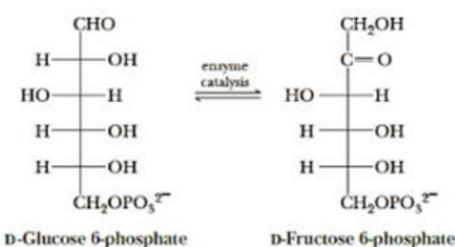


It is an isomerisation. This reaction is important because in this way the hydroxylic group of glucose at C1 is not a pure hydroxylic group because it is an hemiacetal group. For this reason, it is difficult to phosphorylate. This OH derives from the reaction of aldehydic group of glucose with hydroxyl group. Note that the OH at C1 is near an oxygen. Then is more unstable and more reactive than the pure hydroxyl groups. So the isomerization is important because in this way the OH at C1 is a pure hydroxyl group and so it can be better phosphorylated.

The reaction is near to equilibrium at standard conditions (ΔG°) but during glycolysis, some reactions are irreversible (pushed back). Continuously we have a consumption of the products, and when glycolysis is active, the reaction is pushed versus the formation of fructose 6-phosphate.

This and other reactions in glycolysis are reversible so in gluconeogenesis some in these reactions can occur in the opposite way, depending on the regulation.

During the isomerization, an aldose is transformed in a ketose.



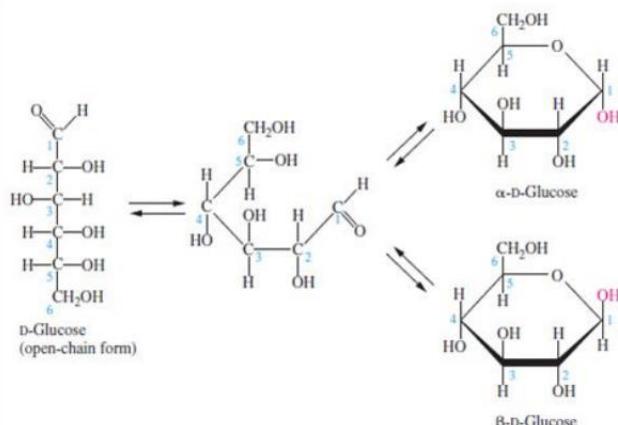
Cyclization of glucose

Why the OH group at C1 in glucose is not a pure hydroxyl group?

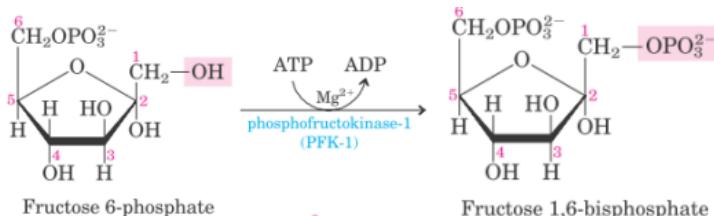
You can see that during the cyclization of glucose, the aldehydic group reacts with OH at C5, to form an hemiacetalic group, which is near an oxygen and has different reaction characteristics compare to a pure hydroxyl group.

Remember that we can have two isomers of glucose due to cyclization:

- Alpha-D-glucose: OH at C1 is down. Gives us the formation of glycogen or starch (used for storage of glucose).
- Beta-D-glucose: OH at C1 is above. Gives us the formation of cellulose in fibres.



STEP 3: phosphorylation of fructose-6-phosphate to fructose 1,6-bisphosphate.



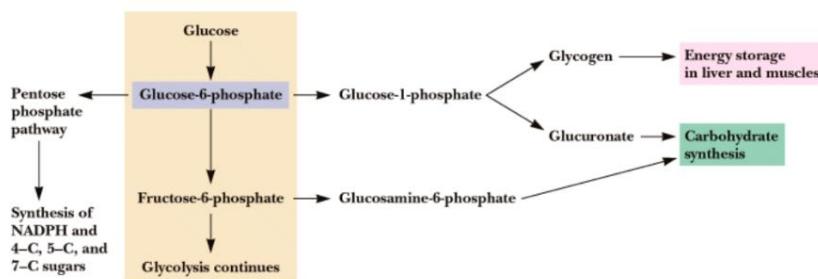
This is one of the most important reactions in glycolysis (remember!).

It is a regulator step. The enzyme that catalyses this reaction is called **phosphofructokinase-1 (PFK-1)**. This kinase phosphorylates the fructose-6-phosphate.

PFK-1 catalyses the second reaction in which ATP is consumed. ATP is hydrolysed to phosphorylate the OH group at C1 to form fructose 1,6-bisphosphate. This reaction is irreversible the delta G is very negative.

Very important reaction because after the formation of the fructose 1,6-bisphosphate, this molecule is committed to go on in glycolysis. And for this reason the enzyme is strictly regulated since after this step the glycolysis is committed to go on and glucose is degraded to form energy.

Why is this reaction important?



Because fructose 1,6-bisphosphate is targeted for glycolysis, instead glucose 6-phosphate and fructose 6-phosphate are intermediates in glycolysis that can be used in other metabolic pathways. The first reaction of glycolysis are in

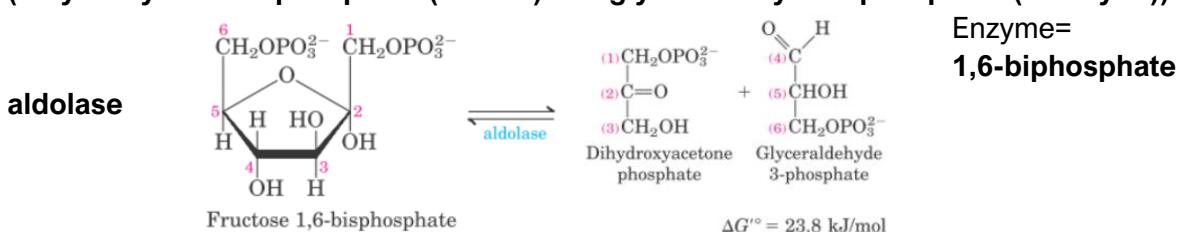
common with other metabolic pathway.

glucose 6-phosphate is at the crossroad of other metabolic pathway such as the pentose phosphate pathway. It will enter in glycolysis if cells need energy.

glucose 6-phosphate can be isomerised into glucose 1-phosphate for synthesis of glycogen (storage of glucose). It is also the precursor for the formation of other sugars that will bring to the carbohydrate synthesis.

For fructose-6-phosphate is the same.

STEP 4: cleavage of fructose 1,6-bisphosphate into two molecules with 3C (dihydroxyacetone phosphate (ketone) and glyceraldehyde 3-phosphate (aldehyde)).



Both of the products are phosphorylated, which is an important aspect because in this way are more reactive for the other reactions.

This reaction is catalysed by an enzyme called aldolase. This reaction at standard condition is endergonic (versus fructose 1,6-bisphosphate) but at actual condition, the glyceraldehyde 3-phosphate is immediately oxidized and the continuous consumption of it shifts the balance to the hydrolysis of fructose 1,6-bisphosphate.

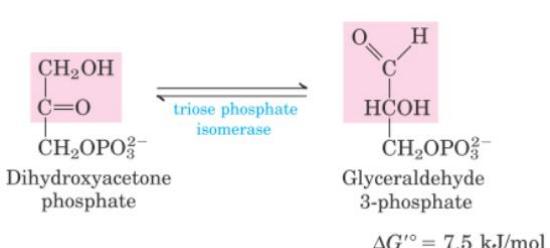
This is an equilibrium reaction so in gluconeogenesis the concentration of the 2 molecules with 3C is higher than the concentration of fructose.

STEP 5: isomerization of dihydroxyacetone phosphate into glyceraldehyde 3-phosphate.

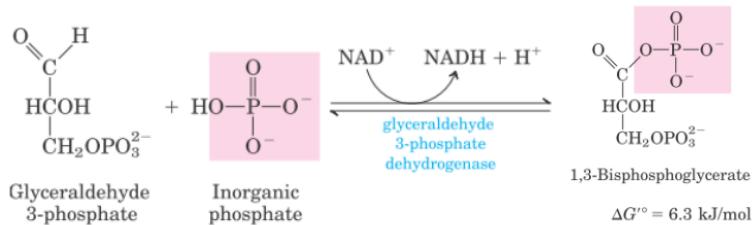
The enzyme is called triose (we have 3C) phosphate (it phosphorylates) isomerase. And the

essential thing to remember is that we have a transformation of a ketone into an aldehyde. The aldehydic group is easier to oxidized into a carboxylic group in the next reaction.

This reaction at standard condition is reversible but in the next reaction we have a redox that drives this reaction versus the products.



STEP 6: Oxidation of glyceraldehyde 3-phosphate to 1,3-bisphosphoglycerate.



Very important because we have the real oxidation of glucose.

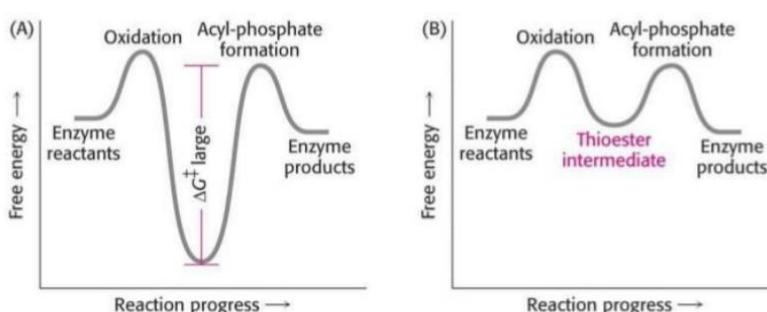
During the oxidation the energy derived is maintained in the product molecule because an inorganic phosphate group binds it to form a molecule with 2 phosphate groups.

We have another product: NADH. During oxidation, electrons are extracted from a molecule and are transported to another one. The acceptor of electrons is NADH⁺, which can acquire two electrons and protons. These electrons can use for reduction or for the synthesis of ATP in mitochondria.

The standard energy is near to equilibrium but the next reaction is exergonic.

Free energy for step 6

The process of glycolysis



by the way

The energy of the 2 coupled reaction have a hole of energy (the first reaction is very exergonic and the second one very endergonic). This enzyme can conserve the energy of the oxidative reaction because in the catalytic site we have the formation of a thioester intermediate that conserves this energy. It is a molecule with high energetic level so the free energy of the reactants is very similar to the thioester. This means that the two reactions have the actual free energy near to 0, so its a reaction that is not very exergonic. But the energy of this thioester is released for the phosphorylation of the glycerate to form products that have more or

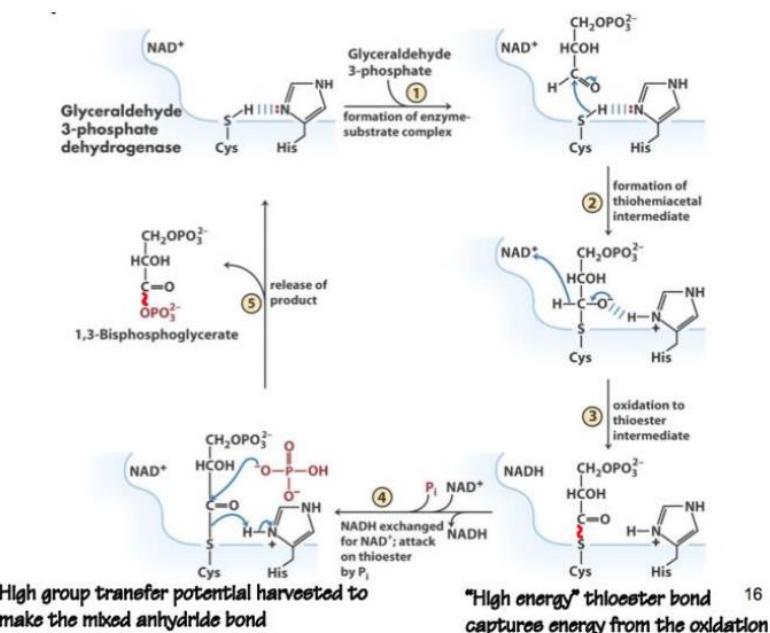
Free-energy profiles for glyceraldehyde oxidation followed by acyl-phosphate formation.

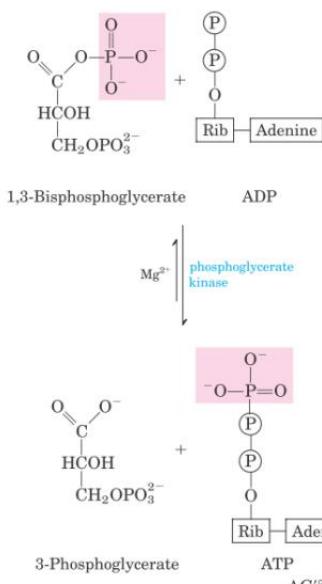
(A) A hypothetical case with no coupling between the two processes. The second step must have a large activation barrier, making the reaction very slow.

(B) The actual case with the two reactions coupled through a thioester intermediate.

less a free energy similar to the reactant. So in this way the enzyme can catalyse the reaction and conserve the energy for producing energy after.

We do not have to remember it.





STEP 7: phosphoryl transfer from 1,3-bisphoglyrate to ADP

One of the two steps in which ATP is synthetised.

1,3-bisphoglyrate has an high energetic bond (P to COOH). The energy derived from the hydrolysis of the phosphate group is used for the formation of ATP starting from ADP, at substrate level.

It is an exergonic reaction.

Enzyme= **phosphoglycerate kinase**

The following two reactions are to prepare the formation of an high energetic substrate:

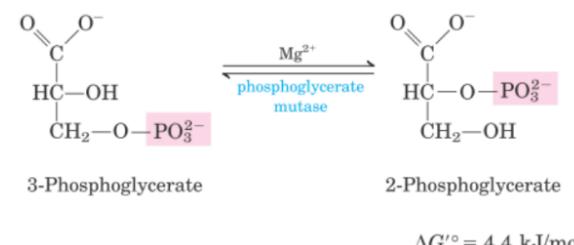
STEP 8: conversion of 3- phosphoglycerate to 2-phosphoglycerate

3-phosphoglycerate is not useful for the synthesis of ATP

directly, need to be modified. The phosphate group is transferred from position 3 to position 2, to form 2-phosphoglycerate.

The reaction is more or less at the equilibrium, so it is reversible but the 2-phosphoglycerate is continuously consumed.

The enzyme is called phosphoglycerate mutase. In this case we do not have an isomerization, we just have a transfer of the phosphate group.



STEP 9: Dehydration of 2-phosphoglycerate to phosphoenolpyruvate.

It is more or less at the equilibrium but phosphoenolpyruvate is very instable and it reacts right away to form a molecule of pyruvate and to form ATP. These two molecules contain nearly the same total amount of energy, but the products are more unstable: the loss of the water molecule from 2-phosphoglycerate causes a redistribution of energy within the molecule, greatly increasing the standard free energy of

hydrolysis of the phosphoryl group.

STEP 10: dephosphorylation of phosphoenolpyruvate into pyruvate.

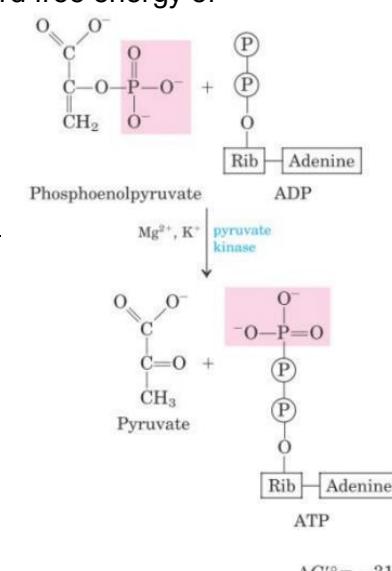
!Remember the pyruvate structure.

Pyruvate is the last molecule produced by glycolysis. The dephosphorylation is very exoergic (look at the delta G) and so irreversible. This reaction drives all the other reactions that are near to the equilibrium because we have the end the synthesis of pyruvate that is very exoergic.

So at the end we have a drop of energy during glycolysis that drives all the other reactions.

It is another example of synthesis of ATP at substrate level because in this enzyme the energy of the dephosphorylation is used for the synthesis of ATP.

The name of the enzyme is pyruvate kinase, which is due to the reverse reaction: it can occur only if we hydrolyse ATP.



7-glycolysis and gluconeogenesis

HOW CELLS OBTAIN ENERGY FROM FOOD: GLYCOLYSIS

- Glucose occupies a central position in metabolism of animal, plants, fungi and microorganisms
- The complete oxidation of 1 mole of glucose to carbon dioxide and water is associated to a standard free energy change of

2840kJ/mol 2840kJ = Energy consumed by a 60Kg man to run about 15Km at a speed of about 10Km/h

1mole of glucose = 180gr

35 gr glucose 600 KJ (3Km)

- Glucose is a versatile precursor for giving metabolic intermediates for biosynthetic reactions such as the carbon skeletons for every amino acid, nucleotide, coenzymes, fatty acids, or other metabolic intermediates.
- Organisms that do not have access to glucose from other sources must make it:

PHOTOSYNTHESIS

GLYCOLYSIS

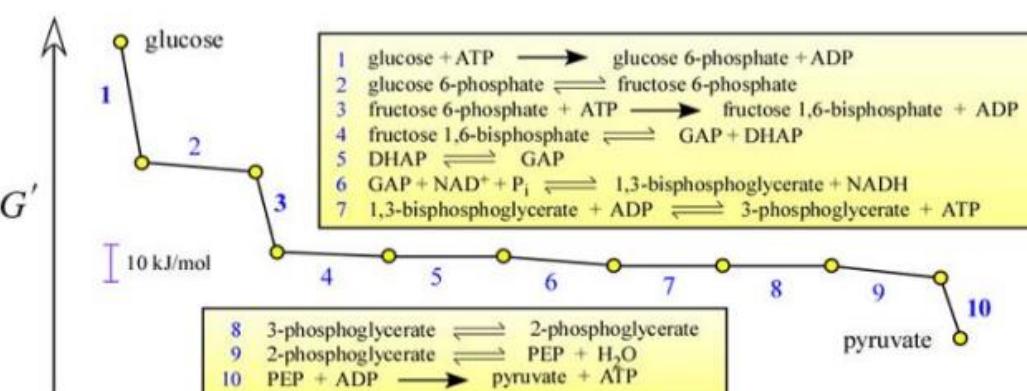
- Glycolysis → from the Greek glykys, meaning “sweet,” and lysis, meaning “splitting”.
- It is the most ancient metabolic pathway
- It is the sole source of metabolic energy in some cells (erythrocytes, brain, anaerobic microorganisms)
- It happens in the cytosol
- A molecule of glucose is degraded in a series of enzyme-catalyzed reactions to yield two molecules of the three-carbon compound pyruvate.

OVERALL ENERGETIC BALANCE OF GLYCOLYSIS

We have ten reactions, in the first part of glycolysis we have two molecules of ATP, the reactions that we can see in the image (1-3) are exergonic because we have the hydrolysis of ATP, we have

coupled reactions in which glucose is phosphorylated using the energy derived from hydrolysis. (1-3)

The change free energy is negative and the reaction 1-2 are irreversible and in nucleogenesis we see that are bypass by other reactions and catalysed by different enzyme; the last reaction (10) is exergonic and in this



case, we will see the bypass of this reaction by different reaction and enzyme.

All the other reactions (4-9) are near to equilibrium so depending on the activation of glycolysis or the activation of nucleogenesis, they can shift depending on the energetic condition of cells or depending on the regulation of the two metabolic pathways.

Many carbohydrates besides glucose meet their catabolic fate in glycolysis, after being transformed into one of the glycolytic intermediates. The most significant are the storage polysaccharides glycogen and starch; the disaccharides maltose, lactose, trehalose, and sucrose; and the monosaccharides fructose, mannose, and galactose. All this sugars are modified to enter in glycolysis, for example galactose is activated but need 3 additional reactions to be modified

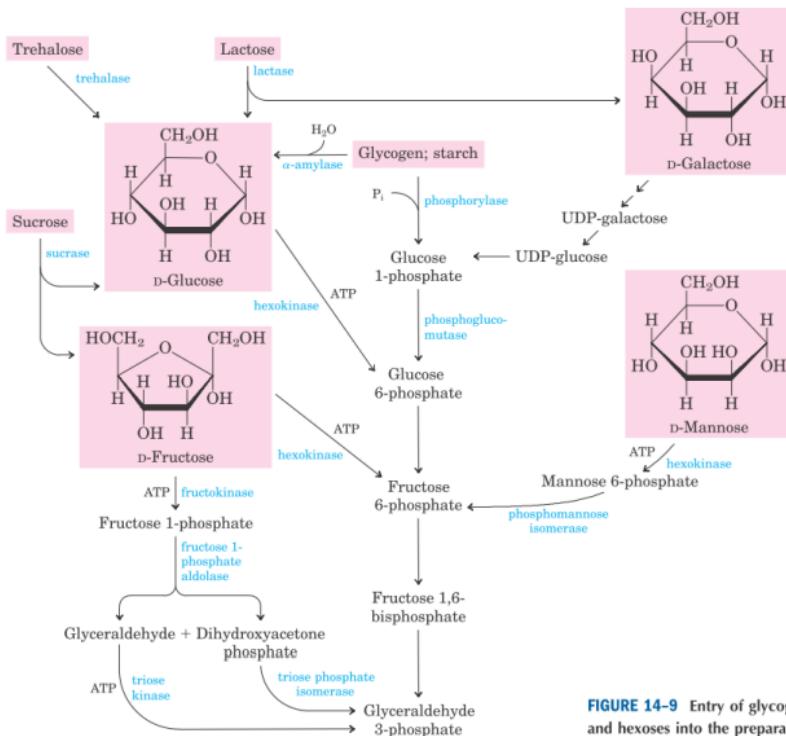


FIGURE 14-9 Entry of glycogen, starch, disaccharides, and hexoses into the preparatory stage of glycolysis.

8-Glucose storages metabolism

Reciprocal regulation of glycolysis and gluconeogenesis

These are metabolites that regulates at the level of allosteric regulation, in fact they interact with allosteric sites in phosphofructokinase-1 and in fructose-1,6-bisphosphatase. This is the most regulated reaction of glycolysis. As we have seen citrate, ATP and protons are inhibitors of phosphofructokinase 1. In general the regulation of glycolysis and gluconeogenesis depends on the energetic demands, so if cells have a lot of energy then PFK-1 is inhibited (by ATP, protons or citrate) to stop glycolysis.

So, when Citrate is accumulated, then it inhibits glycolysis, but at the same time it is an activator of gluconeogenesis (because when we have accumulation of citrate it means that we have a lot of energy and so gluconeogenesis needs to be performed to produce glucose).

Some metabolites are inhibitors of one reaction and activators of the other (like AMP and citrate, which have opposite effects in glycolysis and gluconeogenesis)

Fructose-2,6-bisP is an activator of glycolysis and inhibitor of gluconeogenesis, and it is synthetized by an enzyme, which is regulated at hormonal level.

Regulation of fructose 2,6 bisphosphate levels

The enzyme which regulates Fructose-2,6-bisphosphate has 2 different catalytic activities:
 > acts as a kinase > **PFK2** (kinase domain)

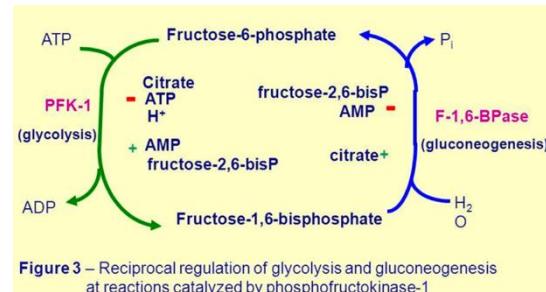


Figure 3 – Reciprocal regulation of glycolysis and gluconeogenesis at reactions catalyzed by phosphofructokinase-1 and fructose-1,6-bisphosphatase.

= allosteric inhibition; + = allosteric activation

>acts as a phosphatase > **FBPase-2** (phosphatase domain)

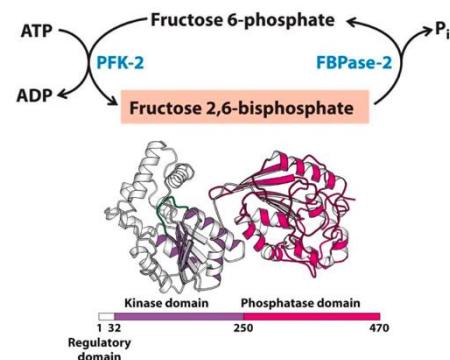
PFK-2 and FBPase-2 are two distinct activities of a single, bifunctional protein

Fructose 2,6 bisphosphate is produced by phosphofructokinase-2 (PFK-2) and broken by fructose 2,6 bisphosphatase (FBPase-2).

In fact, when the PFK-2 is active, it can react with Fructose 6-phosphate and ATP to phosphorylate Fructose 6-phosphate and form Fructose 2,6-bisphosphate.

Reminder > Fructose 2,6-bisphosphate is not an intermediate of glycolysis (the intermediate is Fructose 1,6-bisphosphate), but it derives from an intermediate of glycolysis that is Fructose 6-phosphate.

On the other side, when FBPase-2 is active, the enzyme can perform the opposite reaction and so it can dephosphorylate Fructose 2,6-bisphosphate to form Fructose 6-phosphate.



Regulation of fructose 2,6 bisphosphate levels in liver

When this enzyme is phosphorylated, its structural conformational allows the activity of the phosphatase and inhibits the activity of the kinase.

On the contrary, when this enzyme is not phosphorylated, the kinase is active, and the phosphatase activity is inhibited.

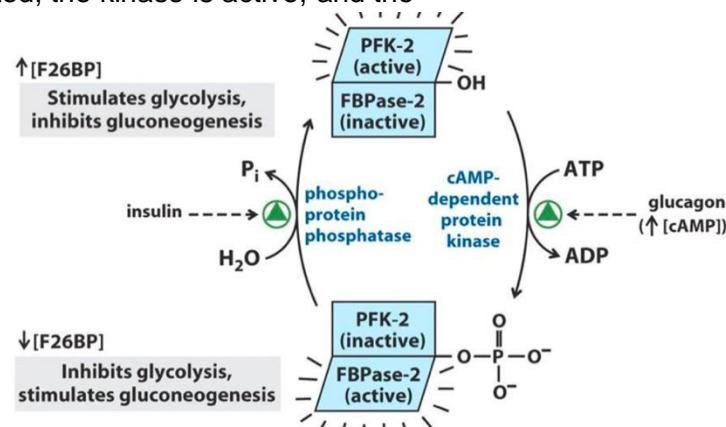
This enzyme is phosphorylated or not depending on the activity of another kinase: **protein kinase A (PKA)**, which is a kinase activated after the production of cAMP.

In fact, when PKA is active, it phosphorylates the enzyme and so the glycolysis is inhibited, and the gluconeogenesis is activated.

- The hormone that activates PKA is Glucagon:

Glucagon is a hyperglycemic hormone, so it is produced by our pancreas when the concentration of glucose in our blood is low. Only liver's cells have the receptor for glucagon (Only liver can react to the production of glucagon), because liver is supposed to produce glucose for maintaining the level of glucose in blood. So, liver has to stop glycolysis (because it has to spare glucose to the other organs) and start gluconeogenesis. To do this, glucagon activates a signal transduction with the production of cAMP, which activates PKA, which then phosphorylates the enzyme, increasing its phosphatase activity and inactivating the kinase activity.

- When in blood we have high glucose levels, liver has to uptake and storage glucose and inhibit gluconeogenesis. In this case glycolysis is activated and gluconeogenesis inhibited. How: **Insulin**, which is an hypoglycemic hormone, produced also in pancreas, stimulates the uptake of glucose in liver and also stimulates the activity of a protein phosphatase, that can dephosphorylate our enzyme and so activate PFK-2 and inhibit FBPase-2



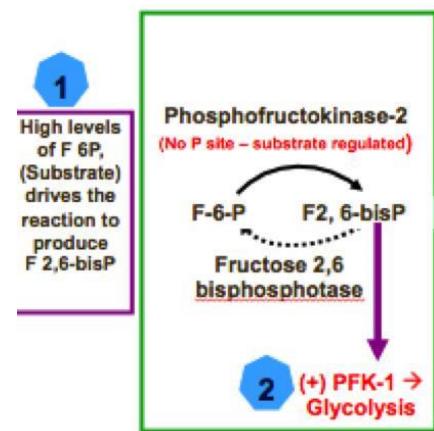
Regulation of fructose 2,6 bisphosphate levels (muscles)

Also in muscles, fructose 2,6-bisphosphate can increase glycolysis (in this case only glycolysis) in a different way.

In this case the enzyme phosphofructokinase-2 is not regulated by phosphorylation, however it can be activated allosterically by fructose 6-phosphate (F-6-P). So, when glycolysis is very active, fructose 6-phosphate is produced at high concentrations and a little part of this metabolite can activate the activity of phosphofructokinase-2, and in this way this enzyme can synthesize fructose 2,6-bisphosphate. And the production of fructose 2,6-bisphosphate, completely activates the PFK-1, increasing the velocity of glycolysis.

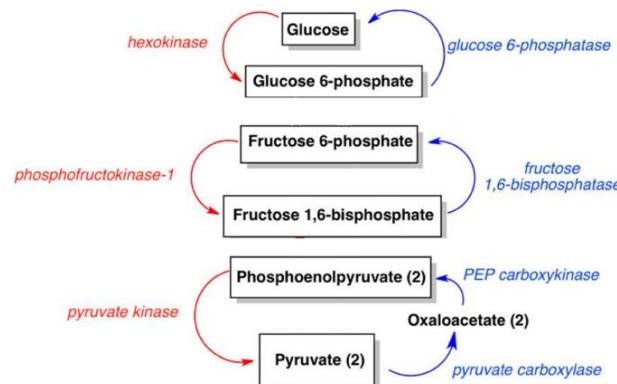
This is an example of feedforward reaction.

Also, in this case the regulation is not at hormonal level, but only at substrate level.



Coordinated regulation of glycolysis and gluconeogenesis

Now, we have the last enzymes to be regulated: in glycolysis pyruvate kinase, and in gluconeogenesis essentially pyruvate carboxylase and a little bit phosphoenolpyruvate carboxykinase.



Pyruvate kinase regulation

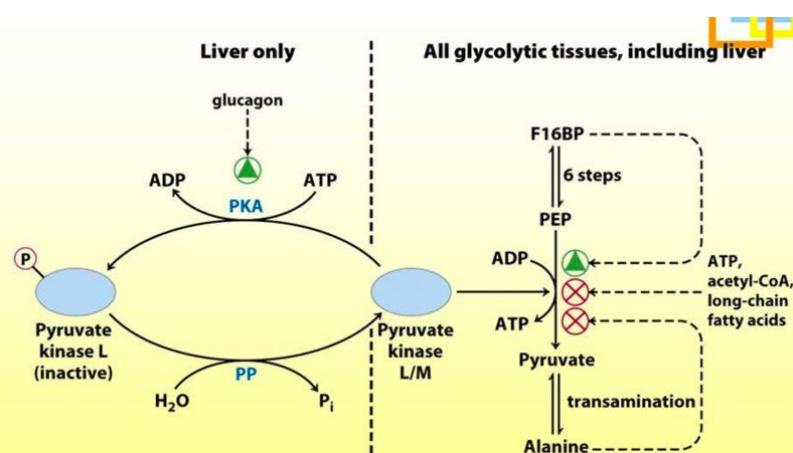
Pyruvate kinase is regulated at substrate level, with allosteric metabolites, and only in liver it is regulated at hormonal level.

-regulation at allosteric level (for all cells)

In all glycolytic tissues, including liver, pyruvate kinase is allosterically activated by fructose 1,6-bisphosphate.

As we know, the reaction catalyzed by pyruvate kinase is one of the most exergonic reactions, so activating this reaction all the other reactions of glycolysis near the equilibrium are shifted versus the formation of pyruvate.

There are also 2 inhibitors of pyruvate kinase: some derive from fatty acids metabolism: acetyl-coA and long chain fatty acids, another inhibitor is alanine, which derives directly from pyruvate and is an inhibitor because if it accumulates it means that cells have a very



active glycolysis, which can be dangerous for them.

-regulation at hormonal level (in particular in liver)

Again, glucagon is synthesized when we are fasted, so our concentration of glucose in blood is low, and glucagon activates PKA by the formation of cAMP. Then pyruvate kinase in liver is phosphorylated by PKA, and so it becomes inactive, and glycolysis is inhibited.

Regulation of pyruvate carboxylase

Pyruvate carboxylase can be the first enzyme of gluconeogenesis, if gluconeogenesis starts from pyruvate (because it can also start from other intermediates).

So, the pyruvate carboxylase is activated allosterically by acetyl-CoA, because acetyl-CoA, when accumulated, indicates that we have a lot of energy, so cells are supposed to have enough ATP to synthesize glucose.

(The carboxylation of pyruvate into oxaloacetate is also important in mitochondria)

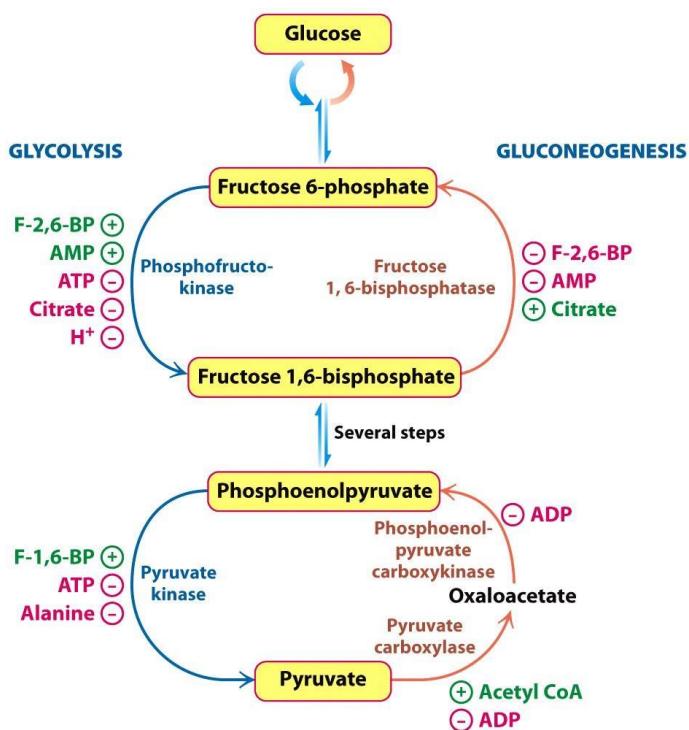


Figure 16.30
Biochemistry, Seventh Edition
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Reciprocal regulation of glycolysis and gluconeogenesis

(ha detto di sapere benissimo questa foto)

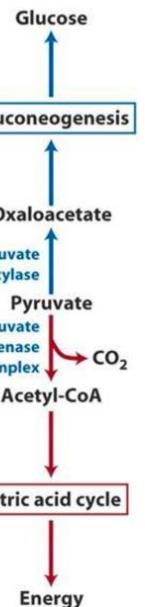
This summarizes the regulation at the allosteric level of glycolysis and gluconeogenesis.

22

But with this photo you can also explain hormonal level (from fructose 1,6-bisphosphate).

In this photo the 2 most important steps that are regulated are shown, but we know that we have also regulation at the level of hexokinase and of glucose 6-phosphate phosphatase. Blue and orange= most important regulated enzymes pink=inhibitors green=activators

Also remember that, in general, low energetic levels activate glycolysis and high energetic levels activate gluconeogenesis.



Metabolism of glucose storages

Glucose can be stored in cells by the formation of polymers, like starch in plants and glycogen in animals. We have also another polymer formed by glucose that is cellulose, but it is a molecule used for the formation of plant's walls and so cannot be digested by animals (only some animals can digest it thanks to some bacteria in their stomach)

Source	Cellulose	Starch		Glycogen
	Plant	Amylose	Amylopectin	Animal
Subunit	β -glucose	α -glucose	α -glucose	α -glucose
Bonds	1-4	1-4	1-4 and 1-6	1-4 and 1-6
Branches	No	No	Yes (~per 20 subunits)	Yes (~per 10 subunits)
Diagram				
Shape				

α - and β - polymers of glucose

Glucose is present in 2 forms: alpha and beta glucose, depending on the cyclization of glucose:

-alpha-glucose, in which the hydroxyl group in position 1 is under the plan, near the OH in position 2

-Beta-glucose, in which the hydroxyl in position 1 is on the other part of the plan, opposite to the OH in 2

So, when glucose forms polymers, it can form alpha 1-4 or beta 1-4 polymers. When polymers are formed by alpha-glucose the OH in position 2 are all in one part of the plan. On the contrary, in beta-glucose polymers the OH in position 2 are present in opposite positions

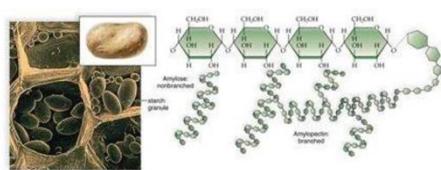
The structures of these polymers are different:

>Alpha polymers: formation of helices and of branches, in the case of branches there is also another bond (1-6)

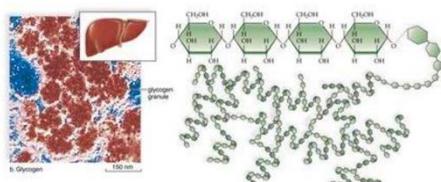
>Beta polymers: we have the formation of fibers

Glycogen and starch structure

Starch structure

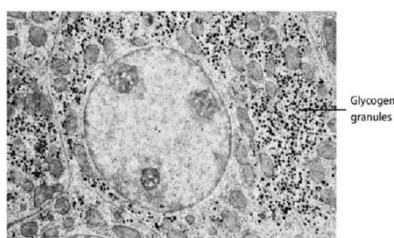


Glycogen structure



because the glucose is a very soluble molecule (with a lot of oxidryl groups)

Glycogen



The structure of glycogen has a central part from which there are several chains of glycogen with several branches.

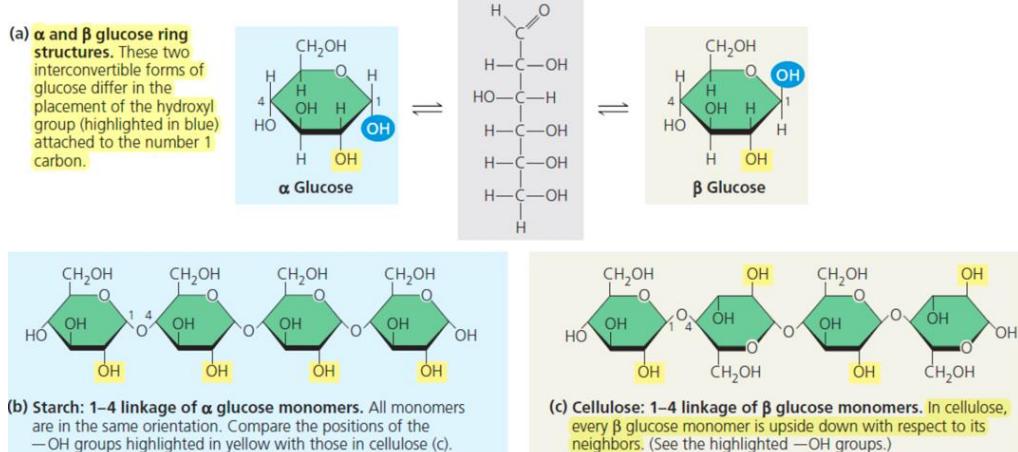
Glycogen is found primarily in the liver and skeletal muscle; it may represent up to 10% of the weight of liver and 1% to 2% of the weight of muscle (in particular red muscles).

Glycogen in muscles is used only for the energy demand of muscles (not of the other organs).

Muscle glycogen can be exhausted in less than an hour during vigorous activity.

Liver glycogen serves as a reservoir of glucose for other tissues, especially for the neurons of the brain, which cannot use fatty acids as fuel.

Liver glycogen can be depleted in 12 to 24 hours.



The important storages of glucose are starch and glycogen. When cells need energy, they can be degraded.

The structures are similar, the only difference are:

>in starch we can have 2 types of starch:

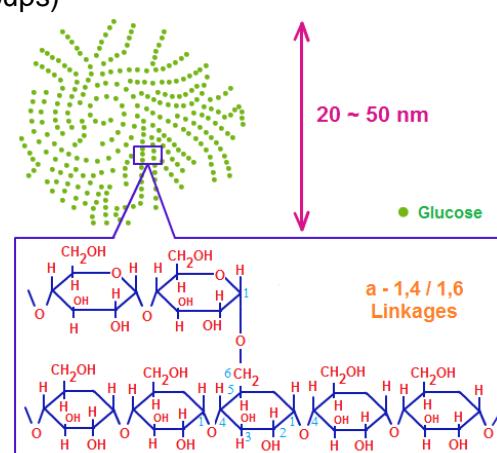
- amylose (only 1-4 polymer)
- amylopectin (both 1-4 and 1-6 bonds)

Whereas glycogen in animals is formed by 1-4 links and 1-6 links

>In starch branches are lower in number compared to glycogen.

Liver is one of the most important organs that stores glycogen.

Glycogen is organized to form granules that are highly hydrated,



Glycogen degradation

Now we start considering the degradation of endogenous glycogen: we analyze the fate of glycogen when cells need energy (they need to produce glucose).

In fact, after the degradation of glycogen, cells produce glucose, which can be used for different metabolic pathways.

The degradation of endogenous glycogen is quite easy because we need only 3 enzymes:

>glycogen phosphorylase

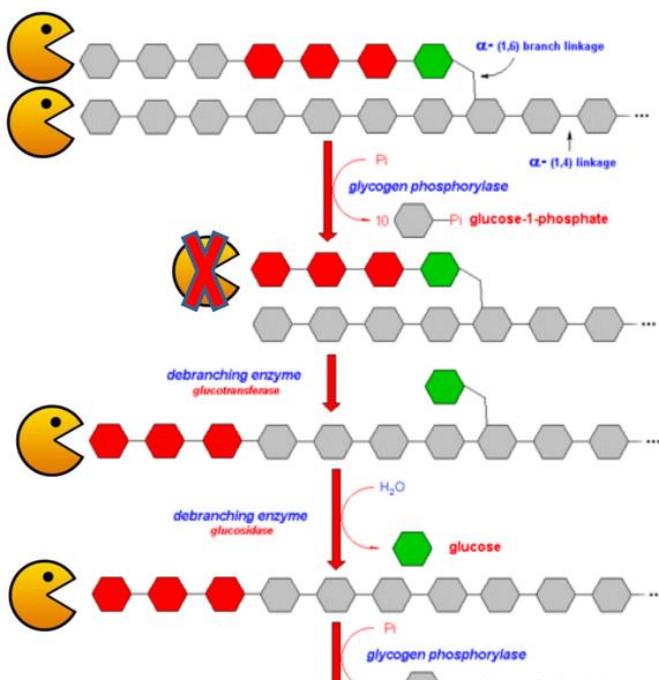
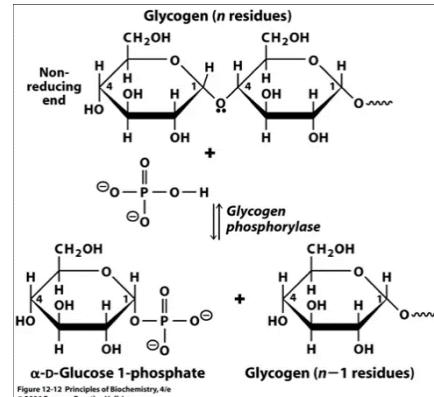
It is the most regulated, and catalyzes the reaction called phosphorolysis reaction (similar to hydrolysis, but the substrate is not water).

The substrate is the phosphate group, and the other substrate is the last glucose in the chain of glycogen (in glycogen there are several chains, and so several glucoses to which the glycogen phosphorylase can attach).

This last glucose is called non reducing end of glucose.

Inside the catalytic site, the glycogen phosphorylase can favor the reaction of phosphate with the 1,4-alpha bond to break this bond and form glucose 1-phosphate and another glucose at the end of the chain.

At the end the chain is shortened of one glucose (which becomes a glucose1-phosphate).



molecule, so glycogen phosphorylase can restart its activity
So, this is a bifunctional enzyme with 2 different catalytic sites for 2 different activities: glucosidase and glucotransferase

In this case, glycogen phosphorylase can phosphorylate the molecules using the energy deriving from the break of the bond, forming glucose 1-phosphate, which is a molecule with a high energetic level.

Glycogen phosphorylase acts repetitively on the non-reducing ends of glycogen branches until it reaches a point four glucose residues away from an (alpha 1→6) branch point, where its action stops.

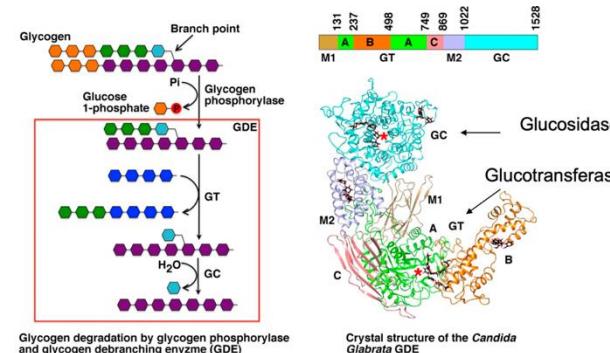
>glycogen debranching enzyme

It is able to do 2 specific reactions:

-transferase activity > break the bonds (1-4) and transfer the molecules of glucose before the 1-6 link to another chain of glycogen.

-glucosidase activity > hydrolysis of 1-6 alpha bond to form glucose.

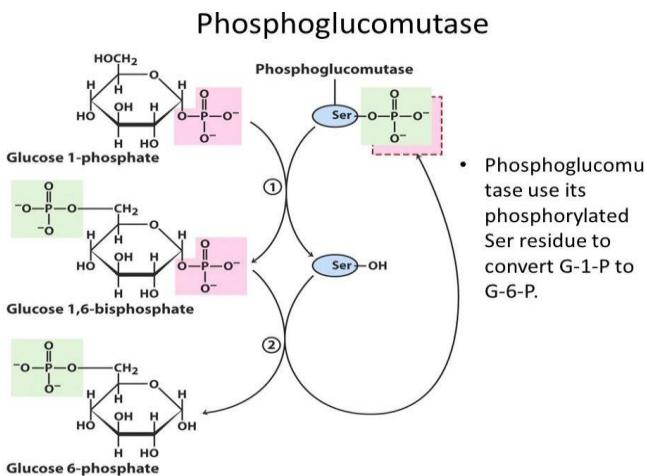
At the end of its action, we have a linear glycogen



>phosphoglucomutase

Glucose 1 phosphate is not a really used molecule in metabolic pathways, whereas glucose 6-phosphate is at the crossroad of different metabolic pathways.

So, glucose 1-phosphate needs to be modified to become glucose 6-phosphate.

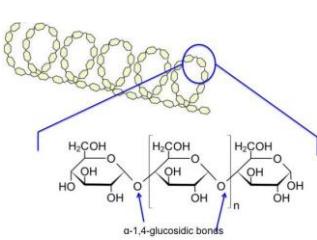


In the catalytic site of phosphoglucomutase we have a serine, which is phosphorylated to phosphoserine, and this phosphate group is important for the formation of glucose 6-phosphate.

In fact, inside the catalytic site, the phosphate from serine is hydrolyzed and the energy deriving is used for the phosphorylation of glucose 1-phosphate and there is the formation of an intermediate, called glucose 1,6-bisphosphate.

Then there is hydrolysis of the phosphate in position 1 to phosphorylate again serine and to get glucose 6-phosphate.

Starch structure



We are considering the degradation of starch in our body

We don't produce starch but we intake it by diet.

Starch consists of 2 types of molecules:

-amylose: a linear molecule, which acquires an helical structure

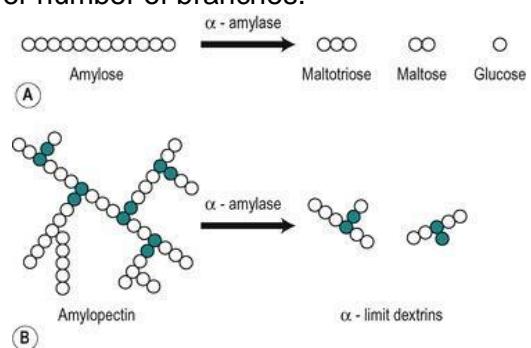
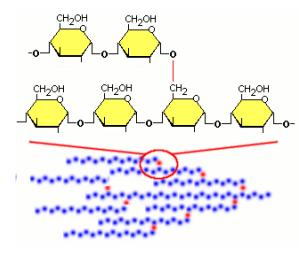
-amylopectin : a branched molecule, which is very similar to glycogen, the only difference is that in amylopectin we have a lower number of branches.

Starch degradation

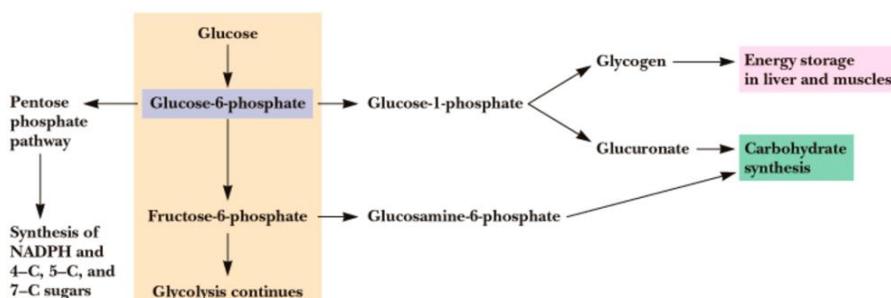
Glycogen and starch ingested in the diet are hydrolyzed by alpha-amylases, enzymes in saliva and intestinal secretions that break (α 1 \rightarrow 4) glycosidic bonds between glucose units.

These enzymes, by hydrolysis, degrade amylose and amylopectin into glucose (or maltose and maltotriose).

In the case of amylopectin, the branched are maintained after degradation, only after they can be degraded by some enzymes able to break the alpha 1,6 bonds.



Glycogen synthesis



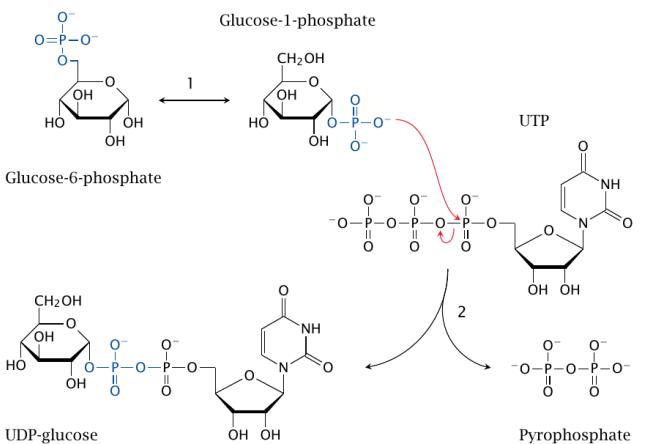
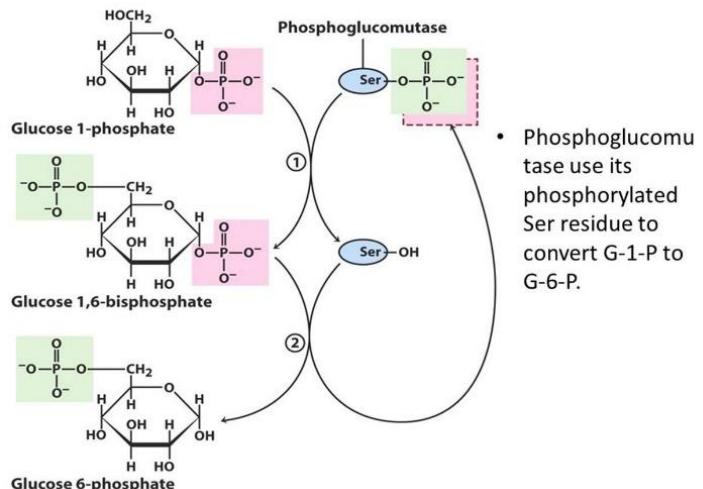
Glucose is absorbed by our intestine and then it can enter the blood. Glucose-6-phosphate is not only a substrate for glycolysis, but it is also an intermediate of other metabolic pathways, like the starch synthesis, the synthesis of different carbohydrates and the pentose phosphate pathway.

When glucose enters in our cells and it is transformed into glucose-6-phosphate (by hexokinase), depending on the energy demands and on the metabolism of our cells, the fate of glucose-6-phosphate can be different.

Phosphoglucomutase

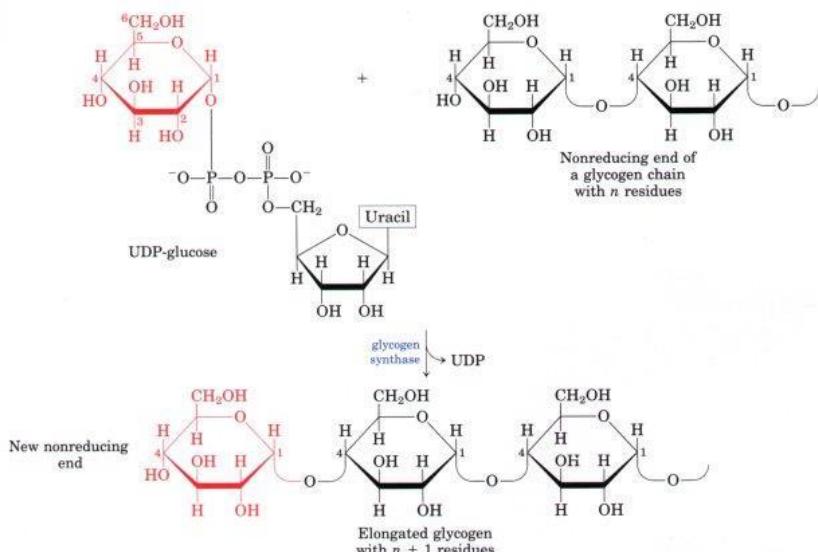
If we look at the glycogen synthesis, we see that the first step is the modification of glucose-6-phosphate into glucose 1-phosphate, which is performed by phosphoglucomutase (it is a reversible reaction)

After the formation of glucose-1-phosphate, it cannot directly be used for the synthesis of glycogen, but it needs to be activated more. So, it



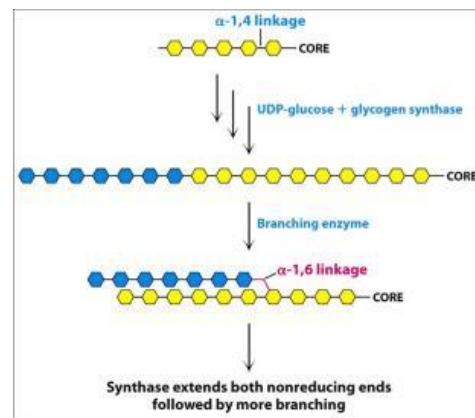
reacts with uridine-3-phosphate (UTP, which is a nucleotide with high energetic level), with the formation of UDP-glucose (uridine diphosphate glucose, a molecule with level of energy similar to ADP, high energetic level). UDP-glucose can then react to synthesize glycogen. The other product of this reaction is pyrophosphate (molecule with 2 phosphates), which is hydrolyzed by pyrophosphatases to drag the whole reaction versus the formation of UDP-glucose.

UTP is generally used to produce energy, and so it is couple with an endergonic reaction. Because when we have intermediates with high energy, often, different nucleotides are required for the formation of these molecules with high energetic level. Usually for sugars the nucleotide used is usually UTP.



UDP-glucose is the substrate of glycogen synthase. Glycogen synthase is the real enzyme that can perform the synthesis of glycogen, so it is regulated. The glycogen synthase favors the reaction of hydrolysis of UDP-glucose and the energy deriving from this hydrolysis is used for the formation of a new alpha 1,4 bond between the last glucose at the nonreducing end of the glycogen chain and the glucose residue from UDP-glucose

Glycogen synthase cannot make the alpha 1,6 bonds for the branching, and so we need another enzyme called glycogen branching enzyme, which is a protein that can break alpha 1,4 bonds, transfer a part of the glycogen chain to another part of the molecule and then synthetize an alpha 1,6 linkage to perform the branching.



9-Pentose phosphate pathway

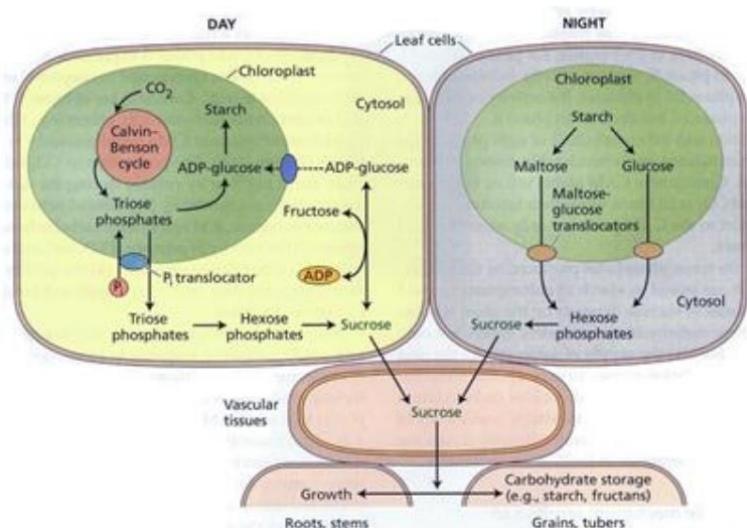
GLYCOGEN SYNTHESIS

Glycogen synthase is the enzyme that adds a molecule of glucose to a chain of glycogen creating the alpha-1,4 linkage. In addition, the branching enzyme is able to transfer a part of glycogen to form branching through the creation of an alpha-1,6 linkage. The glycogen synthase is considered as polymerase infact it can perform its reactions only when it is present a primer, a little chain of glucose, which is produced by a protein called glycogenin. The first step in the synthesis of a new glycogen molecule is the transfer of a glucose residue from UDP-glucose to the hydroxyl group of Tyr194 of glycogenin, catalyzed by the protein's intrinsic glucosyltransferase activity. When we have a little chain of glucose, glycogen synthase can interact with the glycogenin and start adding several glucose. When the chain is long enough, the branching enzyme can start the formation of different branching and several molecules. The glycogen granule has a specific size because the reaction of glycogen synthase goes on until it interact with glycogenin; when the granule is too big they does not interact. Often the glycogen is not completely degraded and the glycogen synthase can act directly on the granule to reduce it. When we finish completely glycogen, the process starts again. The number of glycogenin are genetically defined and so the number glycogen is specific. This is important for the people who play aerobic sports.

STARCH SYNTHESIS

Starch synthesis (performed only by plant cells) occurs, during the day, in chloroplasts for temporary storage of glucose as one of the stable end products of photosynthesis. But during the night, starch is degraded and then glucose and its derived sugars are transported into the non photosynthetic organs.

For long-term storage it is synthesized in the amyloplasts of the non photosynthetic parts of plants (seeds, roots, and tubers).

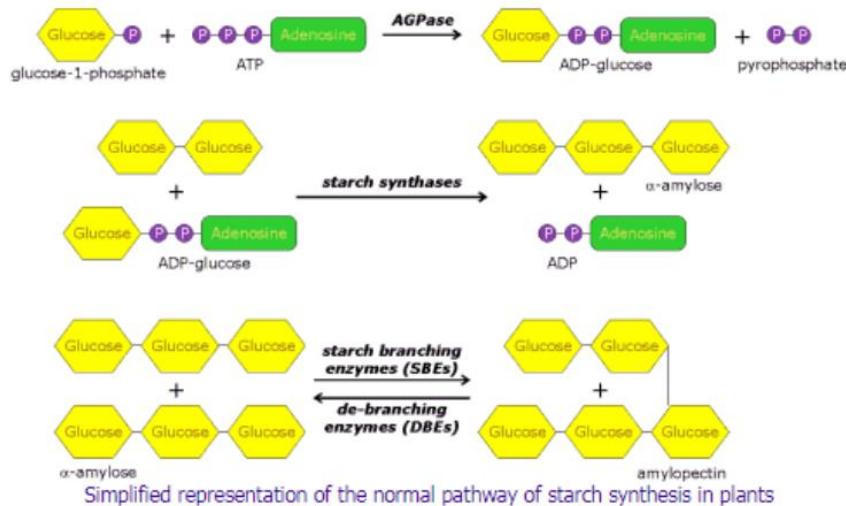


The mechanism of glucose activation in starch synthesis is similar to that in glycogen synthesis.

An activated nucleotide glucose (ADP-glucose) is formed by condensation of glucose 1-phosphate with ATP.

Starch synthase then transfers glucose residues from ADP-glucose to pre-existing starch molecules. The bond is formed using the energy released by the breaking of the bond of ADP-glucose.

The amylose of starch is unbranched, but amylopectin has numerous (α 1 \rightarrow 6)-linked branches. Chloroplasts contain a branching enzyme, similar to glycogen branching enzyme, that introduces the (α 1 \rightarrow 6) branches of amylopectin.



Starch is more compact than glycogen. We cannot store energy only as glycogen (2/3 is water) because our weight will increase.

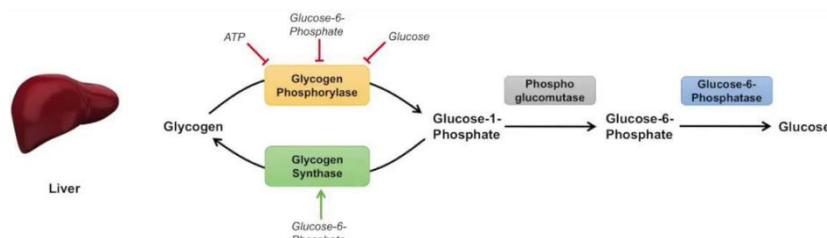
GLYCOGEN METABOLISM REGULATION

The regulation is simpler than the glycolysis and gluconeogenesis ones. In fact the only enzymes that are regulated are glycogen phosphorylase (degradation) and glycogen synthase. They are regulated at allosteric level (allosteric enzymes) and in animals also at hormonal level.

Liver

Glycogen phosphorylase is inhibited by

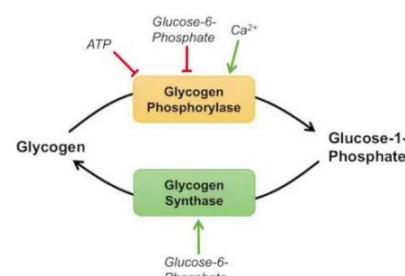
- ATP → if it is present at high concentrations means we do not need energy from glycogen degradation
- glucose-6-phosphate → is one of the indirect product of glycogen degradation. When it is accumulated, means that is not necessary for glycolysis or other pathway. On the contrary, it is an activator of glycogen synthase and synthesis of glycogen is activated inducing the storage of glucose.
- Glucose → high level of glucose, it can enter inside liver (insulin) and it does not need store. (non si capisce un cazzo)



Skeletal Muscle

Glycogen phosphorylase is inhibited by

- ATP
- glucose-6-phosphate



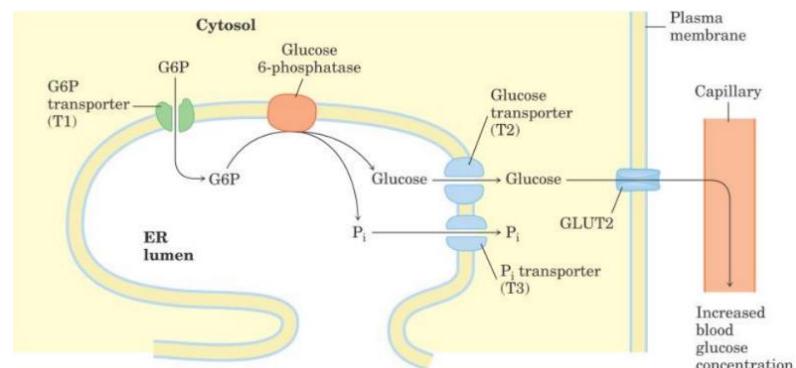
(glucose is not an inhibitor because skeletal muscle is not a regulator of the concentration of glucose in blood)

Glycogen phosphorylase is activated by Ca^{2+} . It is released in cytosol of muscle cells when we have a contraction. So high concentration of calcium in cytosol means that the muscle is active and it is using ATP.

GLYCOGEN METABOLISM REGULATION

In liver the glucose 6-phosphate (G6P), used after the degradation of glycogen and during the synthesis of glucose in gluconeogenesis, is dephosphorylated to glucose in liver to release it in blood circulation. The enzyme involved is glucose 6-phosphatase (present only in liver) which is regulated essentially on the basis on its localization in cells. In fact the active site is faced in human in endoplasmatic reticulum

and G6P has to be transported inside, it is dephosphorylated by the enzyme and transported outside. All these reactions are regulated and the release of glucose in blood is controlled.

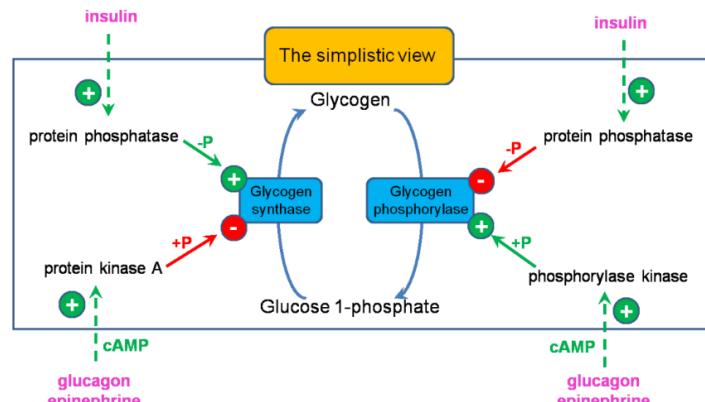


So, as said before the regulation of glycogen in animals depends on the allosteric regulation of activators and inhibitors and on production of hormones, which act at liver and muscle sites. The liver hormones are insulin, hypoglycaemic hormone, glucagon, hyperglycaemic hormone, and epinephrine. The muscle hormone is adrenaline (or epinephrine).

When you produce epinephrine, in muscles glycogen is degraded and at the same time, liver releases glucose in blood for giving energy to the other organs.

So in liver we have receptors for insulin, glucagon and epinephrine while in muscle for epinephrine and insulin, which favours the intake of glucose.

Glycogen synthase is activated when it is dephosphorylated (-P) and inhibited when it is phosphorylated (+P).



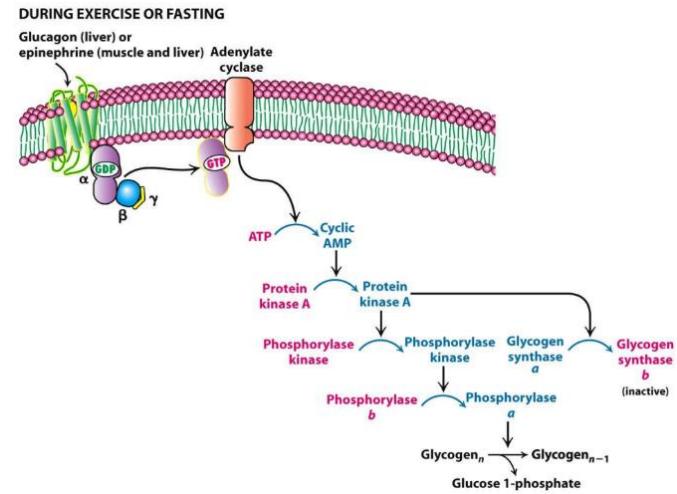
Insulin is an hypoglycaemic hormone, which means that it favours the uptake of glucose in particular in liver cells. Insulin activate a phosphatase that can dephosphorylate the glycogen phosphorylase. Remember that insulin is important for the absorption and storage of glucose in liver cells because liver controls the concentration of glucose in blood. On the contrary, glucagon and epinephrine are hyperglycaemic hormones and both activate the release of glucose in blood through the activation of glycogen degradation and to stop the synthesis of glycogen.

In muscles, epinephrine acts similarly to glucagon. The glucose that is produced by the degradation of glycogen is directly used in glycolysis for the production of ATP. The only difference in this case is that glucose is not released in blood.

How epinephrine and glucagon work.

Liver presents the receptor only for glucagon while muscles for both of them. Several proteins are then activated and at the end we have an increase of cyclic AMP in cytosol, which activates PKA. PKA has several targets:

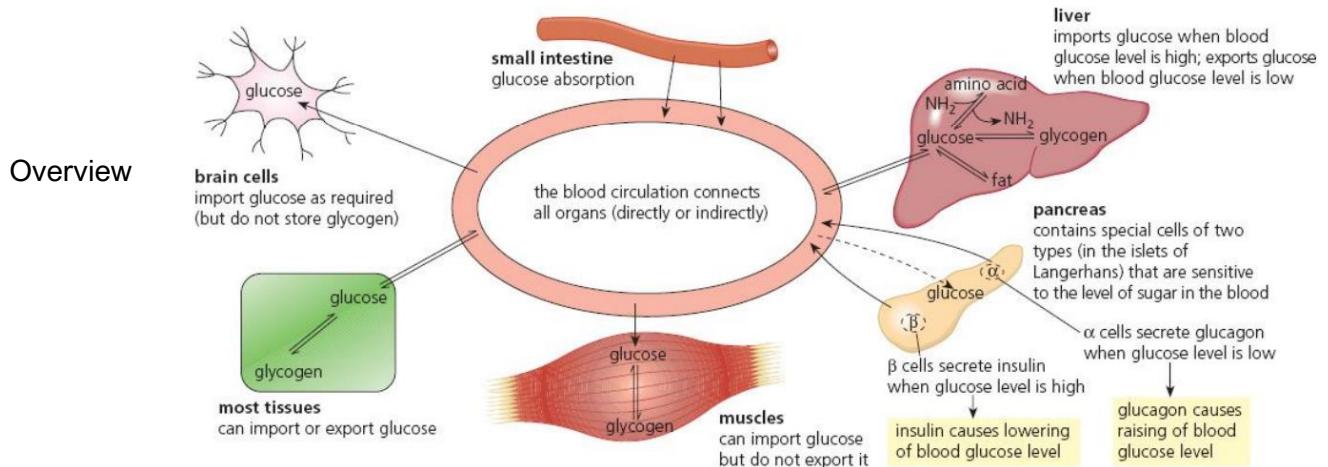
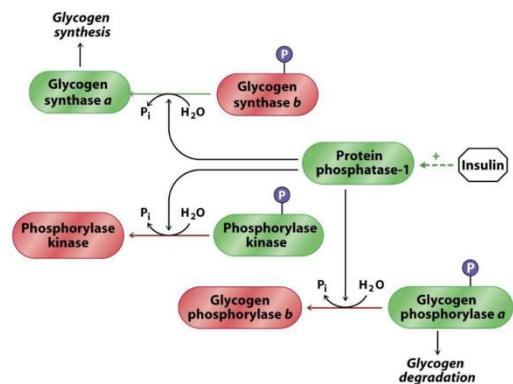
1. glycogen synthase. When it is + P it is inhibited, and it is called glycogen synthase b otherwise glycogen synthase a (-P).
2. activates the glycogen phosphorylase b into glycogen phosphorylase a, but not directly. It is +P by the glycogen phosphorylase kinase which is activated by PKA. Glycogen phosphorylase can degrade glycogen into glucose.



During a signal transduction we have an amplification of the signal, so the presence of glycogen phosphorylase kinase increases the effect of the hormone.

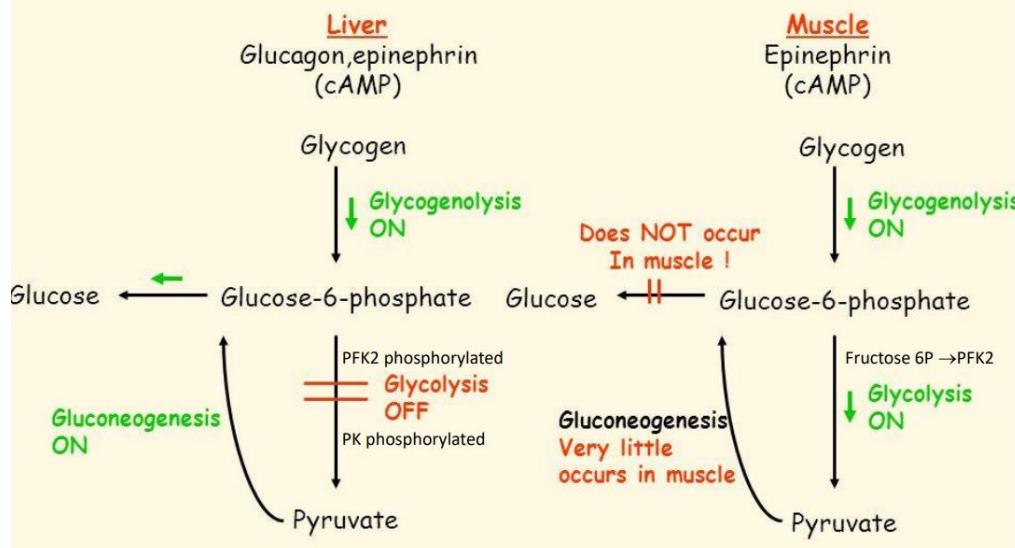
Insulin regulates the metabolism of glycogen at different steps:

- activates phosphatase which acts directly on the glycogen phosphorylase. It -P, inhibiting it and so insulin favours the synthesis of glycogen.
- Inhibits (-P) the phosphorylase kinase.
- Activates glycogen synthase (-P)



Summary

Glycolysis and Gluconeogenesis - regulation - hormonal



Liver

When the hormones are synthesised, glycogen degradation is activated to produce G6P and after glucose. Glucose is not used in glycolysis because the +P of PFK2 inhibiting it and activates the activity of the phosphatase. In this way, the fructose 6-phosphate is not reduced and glycolysis is inhibited and gluconeogenesis is activated. With the same hormones, both the metabolic pathways converge to the formation of G6P.

After the activation of pyruvate kinase, which is the last enzyme of glycolysis and when it is +P, it is inhibited.

Muscles

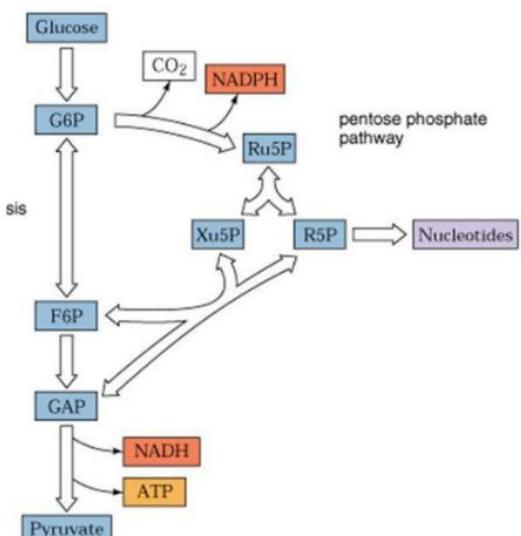
It degrades glycogen to produce G6P, which is immediately used for glycolysis.

The PKA is activated but cannot +P the enzyme that synthesizes fructose 6P, which is an activator of PFK2. It synthesizes fructose 2,6-bisphosphate in an allosteric way. And gluconeogenesis is inhibited.

PENTOSE PHOSPHATE PATHWAY (PPP for friends)

In animals and vascular plants, glucose has three major fates:

- Stored, in glycogen or starch (as a polysaccharide or as sucrose).
- oxidized to a three-carbon compound (pyruvate) via glycolysis to provide ATP and metabolic intermediates.
- oxidized via the pentose phosphate pathway to yield ribose 5-phosphate for nucleic acid synthesis and NADPH for reductive biosynthetic processes.



It is considered at the crossroads of catabolic and anabolic pathways because in the first part we have oxidative reactions (carboxylation and production of NADPH, which is used for anabolic pathways).

In addition, the intermediates that are formed during the PPP are reorganized to form F6P and glyceraldehyde phosphate (GAP), which are intermediates of glycolysis. PPP is so like a branch of glycolysis. So it is not linear pathway since we do not have a starting and a finishing molecule, but it starts and finishes with an intermediate.

! Ribose 5 phosphate (Ru5P) is a sugar that is at the base of synthesis of nucleotides.

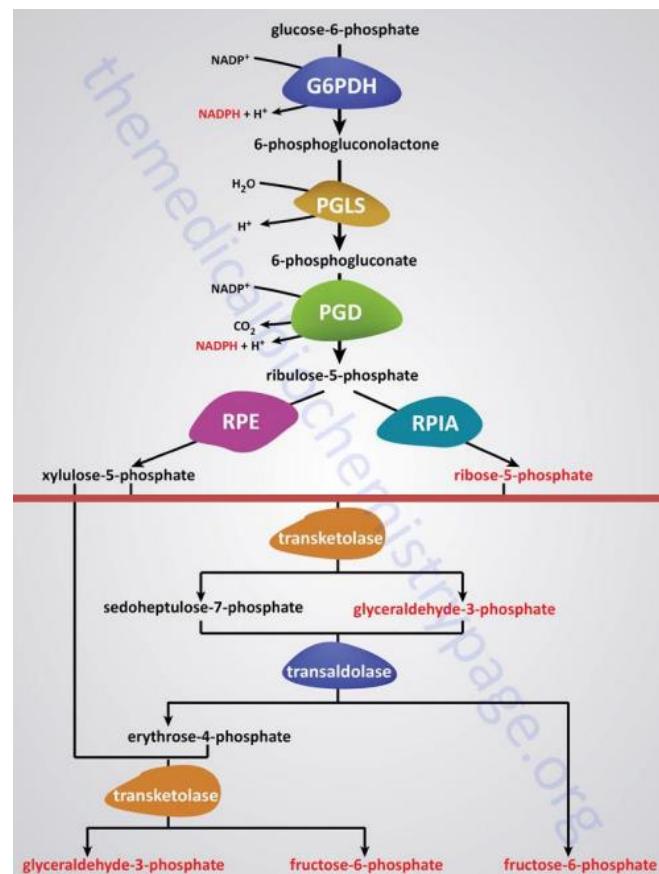
PPP is interconnected with cellular metabolic pathways.

It is in fact very active in cells:

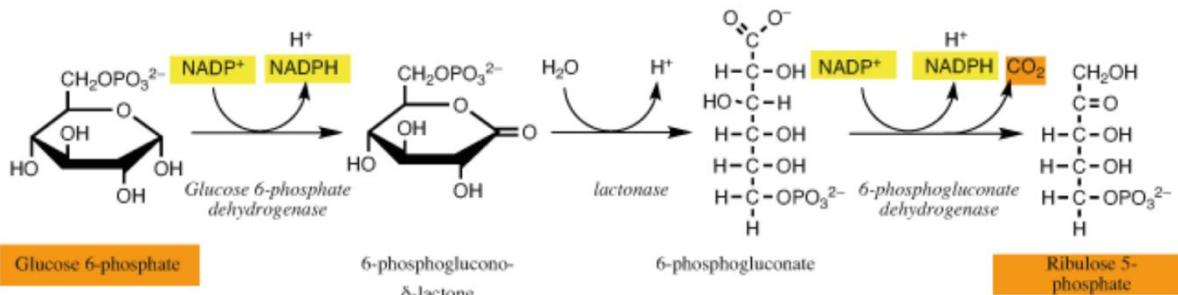
- Rapidly dividing cells, such as those of bone marrow, skin, and intestinal mucosa, use the pentoses to make RNA, DNA, and such coenzymes as ATP, NADH, FADH₂, and coenzyme A.
- Tissues that carry out extensive fatty acid synthesis or very active synthesis of cholesterol and steroid hormones require the NADPH provided by the pathway.
- Tissues in which is important to counter the damaging effects of oxygen radicals, the essential product is NADPH.
- Overview of PPP, which can be divided into parts:
 - Oxidative phase: comprehends the reactions in which NADPH is synthesized, so here we have the oxidative reactions. At the end we have the production of an important molecule which is Ru5P.
 - Non-oxidative phase: several reactions in which the two products of oxidative phase are rearranged, and we can have several intermediates. The most important are at the end: GA3P and F6P. They are important because they can be recycled in glycolysis or gluconeogenesis. We also have two intermediates that can be also intermediates for the synthesis of amino acids.

This pathway can explain very well the interconnections many pathways.

Tissues with active pentose phosphate pathways	
Tissue	Function
Adrenal gland	Steroid synthesis
Liver	Fatty acid and cholesterol synthesis
Testes	Steroid synthesis
Adipose tissue	Fatty acid synthesis
Ovary	Steroid synthesis
Mammary gland	Fatty acid synthesis
Red blood cells	Maintenance of reduced glutathione



OXIDATIVE PHASE



G6P is oxidized to obtain a molecule of 6-phosphogluconolactone. You see the OH group at C1 is oxidized to obtain a COOH group with the formation of a molecule of NADPH.

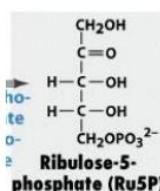
This molecule can now react with a molecule of water and the ring is open to give 6-phosphogluconate, which is oxidized and decarboxylated to form NADPH and ribulose 5-phosphate. This last molecule can be immediately isomerized to give ribose 5-P.

Most important products: NADPH and ribose 5-P.

NON-OXIDATIVE PHASE

Note: the separation in phases is just for teaching, is not a clear distinction.

NON-OXIDATIVE PHASE



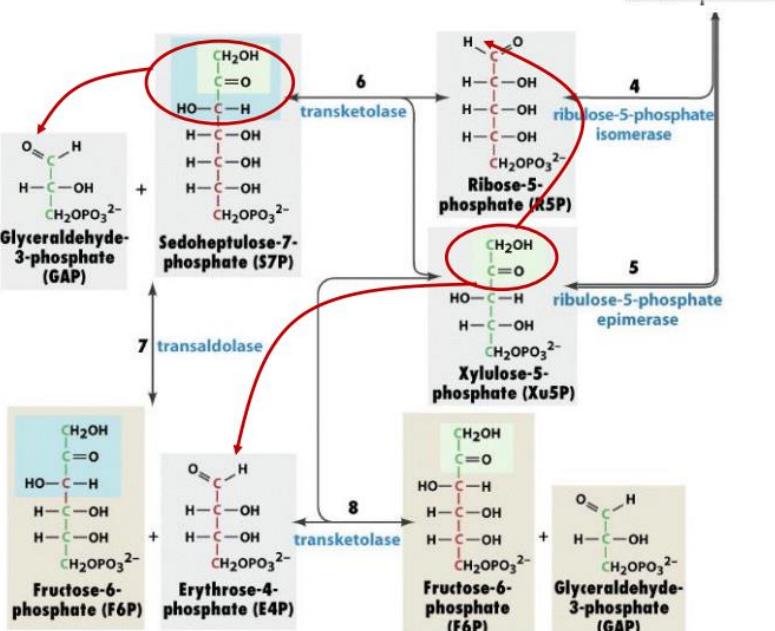
So Rub5P is isomerized to give ribose 5-P and at the same time is modified by an epimerase to give xylulose 5 phosphate (Xu5P). Both molecules have 5 C and are +P.

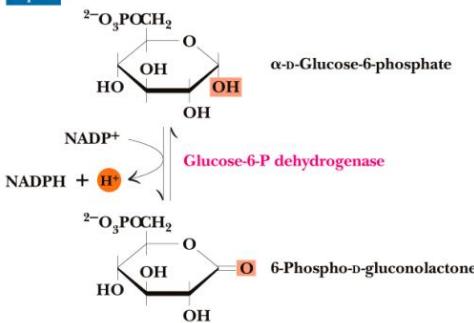
Now we have a rearrangement to form the other phosphorylate carbon hydrates.

At this point, 2 C are transferred from Xu5P to ribose 5-P to form a molecule with 7 C (Sedoheptulose-7-phosphate (S7P)) and the remaining part of Xu5P, which is glyceraldehyde-3-phosphate (GAP). The enzyme that catalyses this reaction is called transketolase because a keto group is transferred to an aldehydic group.

Then transaldolase transfer 3 C from S7P to GAP to form F6P (6 C) and erythrose-6-phosphate (E4P - 3 C). These molecules are intermediates of others metabolic pathways.

Transketolase can transfer from Xu5P to E4P 2 C, with the formation of F6P and GAP.

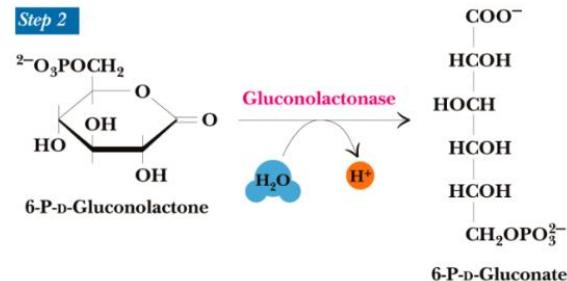


Step 1

STEP 1: G6P oxidation to 6-phosphogluconolactone with the formation of NADPH

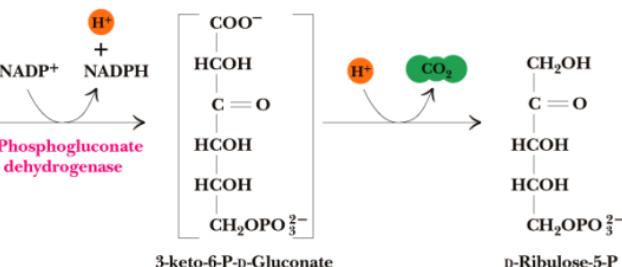
This reaction is catalysed by glucose-6P dehydrogenase. This is enzyme is the most regulated of the pathway since it is the first one. Its regulation depends on the concentration NADP⁺ or NADPH.

The formation of this ketonic group is important because this molecule is induced to be hydrolysed.

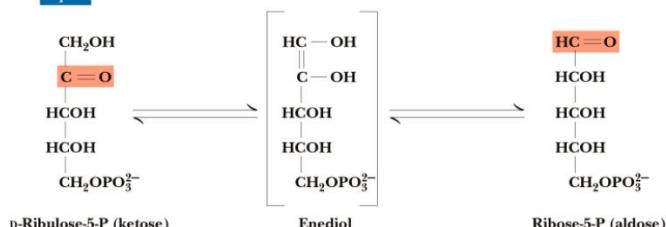
Step 2

STEP 2: hydrolysis of 6-phosphogluconolactone to 6Pgluconate

6-phosphogluconolactone is hydrolysed very easily since the reaction is exergonic. Then we obtain a linear molecule called 6Pgluconate.

Step 3

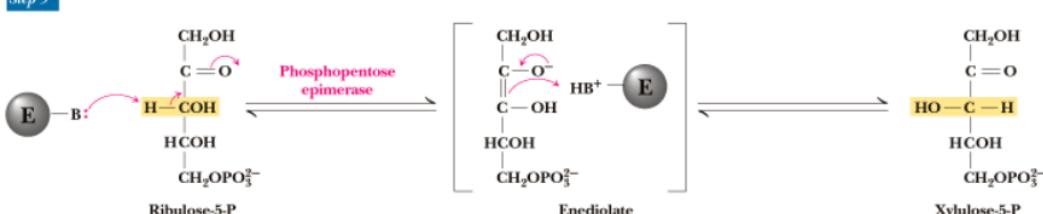
STEP 3: oxidative decarboxylation of 6Pgluconate to Ru5P with the formation of a molecule of NADPH and of CO₂.

Step 4

STEP 5: epimerization of ribose 5P to Xu5P.

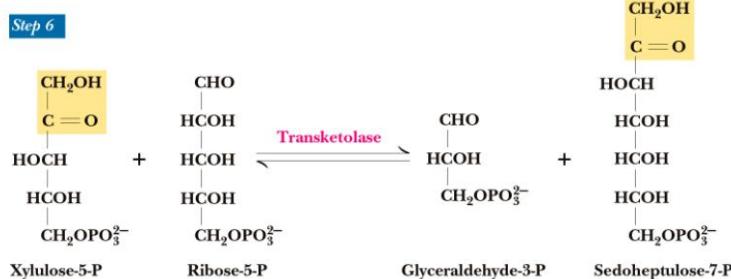
Epimerization is a chemical process where an epimer is made to transform into its chiral counterpart

Note: it is not linear but a branched.

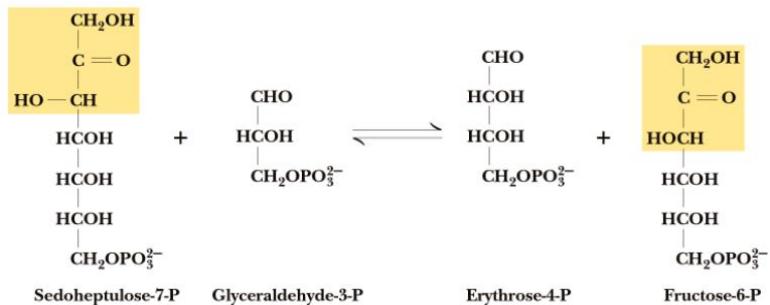
Step 5

STEP 6: rearrangements of Xu5P and ribose 5P carbon skeletons.

Transketolase coenzyme is thiamine pyrophosphate (it derives from vit B1), which is involved in carbon hydrate metabolism.

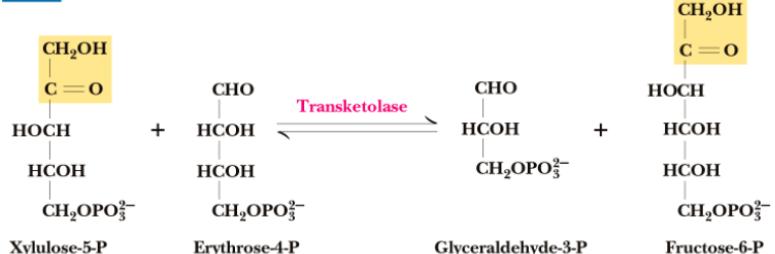


Step 7



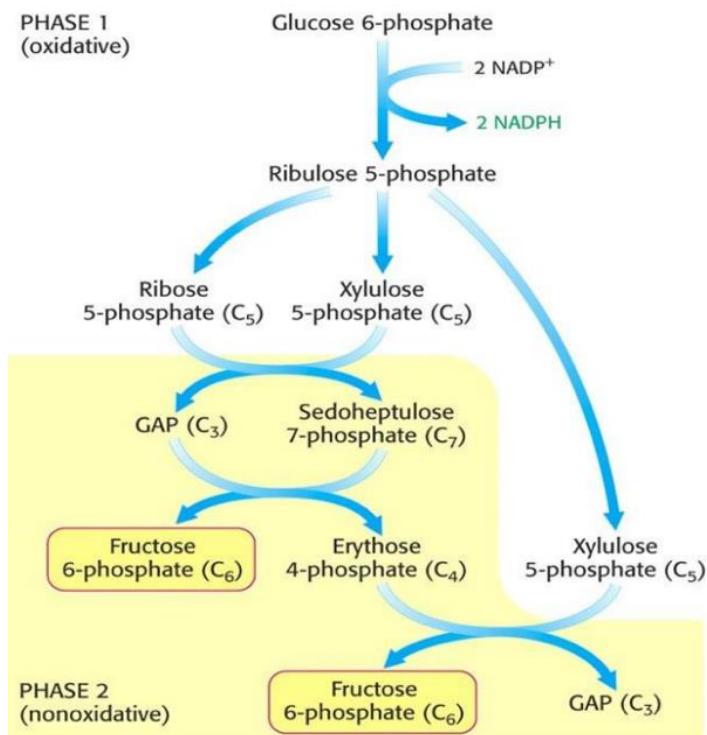
STEP 7: rearrangements of S7P and GAP carbon skeletons to form E4P and F6P.

STEP 8: rearrangements of Xu5P and E4P to form GAP and F6P.

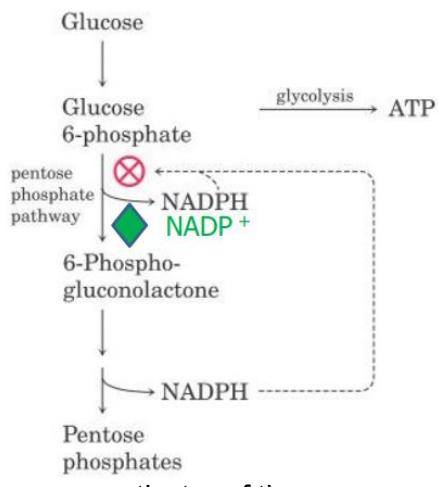


All these reactions are reversible, except the second and the third. This is important because in this way PPP is very flexible.

Summary:



REGULATION OF PPP



The pentose phosphate pathway depends on the current needs of the cell and on the concentration of NADP⁺ in the cytosol.

When a cell is rapidly converting NADPH to NADP⁺ in biosynthetic reductions, the level of NADP⁺ rises, allosterically stimulating G6PD.

When the demand for NADPH slows, the level of NADP⁺ drops, the pentose phosphate pathway slows.

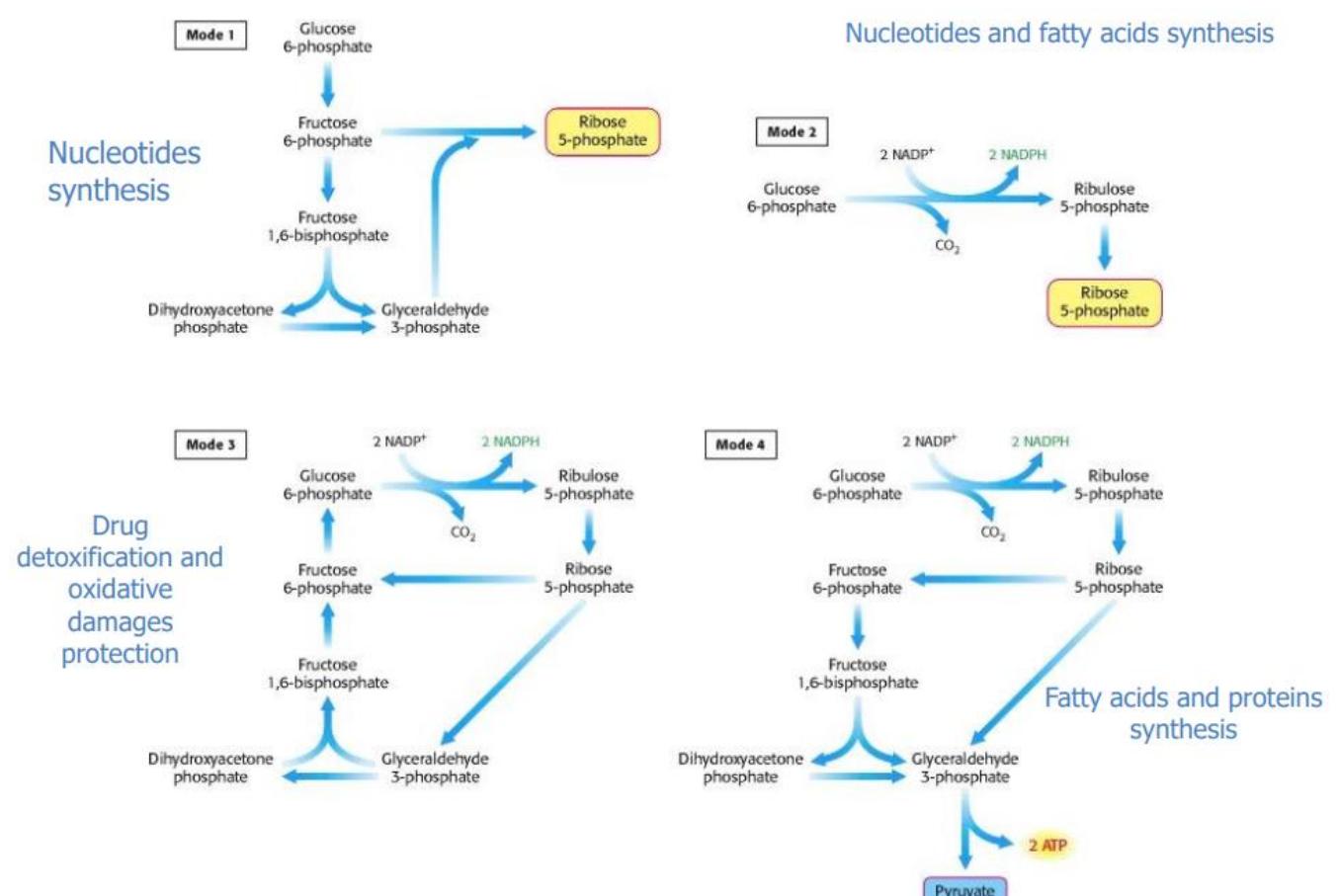
It is regulated by the balance of NADPH and NADP⁺.

When we have a lot of NADPH, it is an inhibitor of G6P dehydrogenase and so inhibits PPP in particular the oxidative phase. That means that the cells need NADPH for biosynthetic pathway and so NADP⁺ is an activator of the enzyme.

It is important to regulate the first part because it is the irreversible part.

So if PPP is inhibited, G6P is accumulated and used glycolysis if cells need energy otherwise it is used in the synthesis of glycogen.

!! Four possibilities of different metabolic pathways in which PPP can shift its reactions depending on the needing of the cell.



1. We have to consider cells in which we have a high activation of nucleotides synthesis. So, the most important product required from PPP is ribose 5P for the synthesis of nucleotides. In this case cells do not require NADPH because they only want to see the tight

nucleotides, and so the oxidative part is inhibited but from glycolysis F6P and GAP can shift versus the synthesis ribose 5P using the reverse reaction of PPP that we have seen.

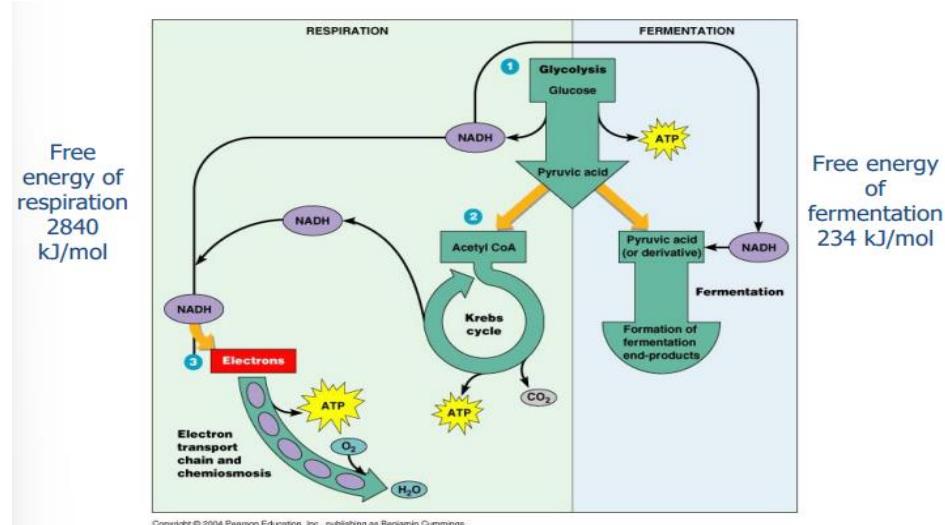
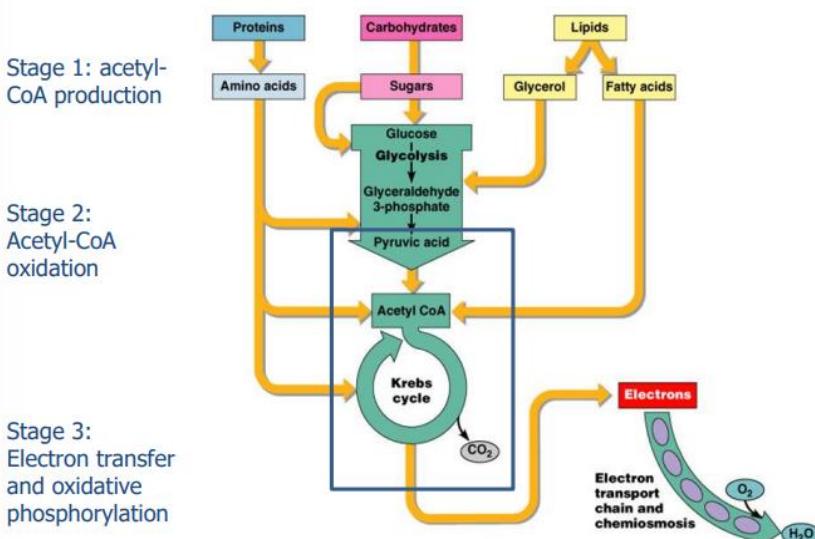
2. In cells, in which nucleotides and fatty acids synthesis are activated (cells that are growing and dividing), need NADPH for the synthesis of fatty acids and ribose 5P for the synthesis of nucleotides. In this case, only the first part of PPP is active because the two products are pushed versus their synthesis and ribose 5P does not need to be rearranged.
3. Cells involved in blood detoxification, occurs often in liver cells. In this case, there is the production of many oxidant radicals and so cells are activating the scavenging pathway for eliminating the radicals, which needs essentially NADPH. So PPP is all active: NADPH from oxidative phase and the intermediates are recycled to enter in glycolytic or gluconeogenic pathway, depending on the necessity of the cell. The pathway is organized to produce at the end F6P and GAP.
4. Cells are growing. They need NADPH for anabolic reactions and energy for the biosynthetic pathway (protein and fatty acids). So the oxidative phase is active for producing NADPH and the fate of F6P is essentially versus the complete oxidation of pyruvate to produce more ATP to go in glycolysis.

10-Aerobic fate of pyruvate-the TCA cycle

THE AEROBIC FATE OF PYRUVATE

Pyruvate is completely oxidized by carbon dioxide and water. Usually, all this process is called respiration. The complete oxidation happens in mitochondria, they produce a lot of energy after the oxidation.

CELLULAR RESPIRATION OCCURS IN THREE MAJOR STAGES



The first part is the introduction of acetyl-CoA production, that is one of the molecules that is centre of the metabolism, is also used to the synthesize of fatty acids and some amino acids.

All the metabolic pathway in aerobic cells produces at the end Acetyl-CoA, that is the first substrate of the second part of the respiration, that is a very important part in cellular metabolism, because in this part we have the oxidation of Acetyl-CoA but in particular the formation of several intermediates and these are useful for metabolic and producing energy.

One of the most important metabolic pathways in aerobic cells is Krebs's cycle. The name of the cycle derives from its discoverer Hans Krebs, and can be called tri-carboxylic acid cycle (TCA) or the citrate cycle.

The most important molecules produced in this cycle are NADH and FADH₂.

Electrons are transferred from these molecules into the respiratory (electron-transfer) chain, the electron transfer chain can transport electrons from NADH and FADH₂ to oxygen, and the energy derives from this redox reaction is used for the synthesis of ATP.

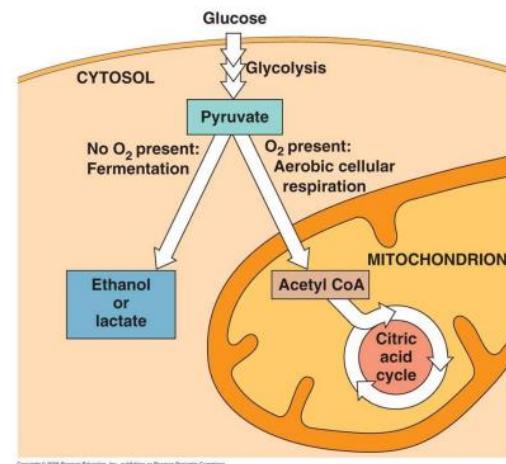
PYRUVATE OXIDATION OCCURS INTO MITOCHONDRIA

We are focusing on the oxidation on pyruvate acetyl-coA.

When is formed we are in cytosol, but the citric acid cycle and the oxidation happen in the mitochondrion.

There is a specific protein that transports pyruvate inside the mitochondrion.

The molecule that enters the Krebs's cycle is acetyl-coenzyme A. Acetyl CoA is produced in the first phase of the aerobic respiration through degradation of the initial chemical substrate. It is used to obtain NADH and FADH₂ during acetyl CoA oxidation and finally the electron transfer and oxidative phosphorylation allows the pathway to use the electrons for the respiratory chain, as mentioned before. Besides acetyl-CoA, any compound that gives rise to a four- or five-carbon intermediate of the citric acid cycle, for example the break-down products of many amino acids, can be oxidised by the cycle



PYRUVATE IS OXIDIZED TO ACETYL-COA AND CO₂

The first reaction inside the mitochondria is the oxidation of pyruvate into acetyl CoA (more precisely, oxidative decarboxylation). This is a complicated reaction, that is catalysed by a multienzymatic complex called PYRUVATE DEHYDROGENASE (PDH):

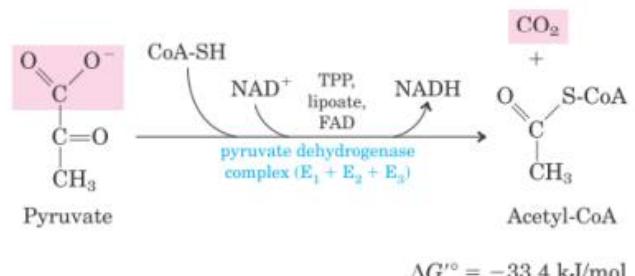
Three enzymes: E1 (pyruvate dehydrogenase),

E2 (dihydrolipoyltransacetylase), E3 (dihydrolipoyldehydrogenase). At the end of oxidation, you obtain a molecule of acetyl CoA, carbon dioxide and NADH. It's an irreversible reaction, so PDH is very strictly regulated

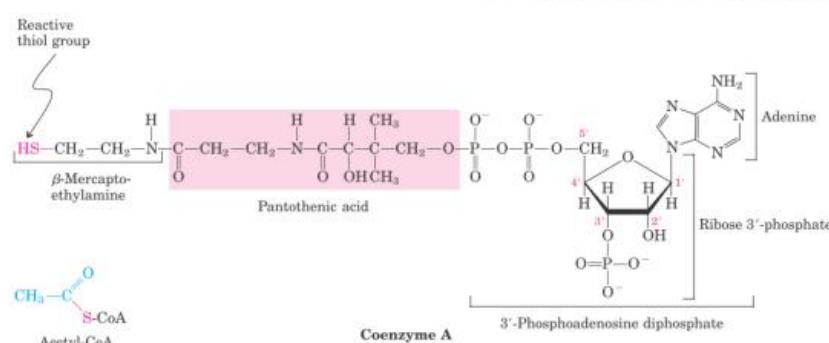
5 cofactors: NAD⁺; Coenzyme A (CoA-SH); thiamine pyrophosphate (TPP); Lipoate, FAD+

Coenzyme A has a thiol group (SH) that

is critical to the role of CoA as an acyl carrier in a number of metabolic reactions. Acyl groups are covalently linked to the thiol group, forming thioesters (very reactive): because of their relatively high standard free energies of hydrolysis, thioesters have a high acyl group transfer potential and can donate their acyl groups to a variety

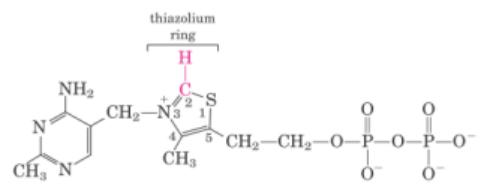


16.1 Production of Acetyl-CoA (Activated Acetate)

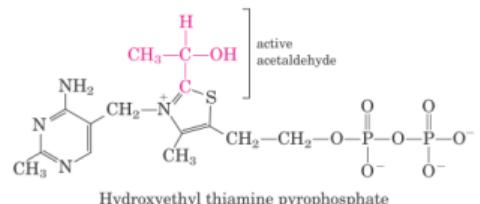


of acceptor molecules. The acyl group attached to coenzyme A may be thought of as 'activated' for group transfer.

Thiamine pyrophosphate (from vitamin B1) plays an important role in the cleavage of bonds adjacent to a carbonyl group, such as the decarboxylation of α -keto acids, and in chemical rearrangements in which an activated acetaldehyde group is transferred from one carbon atom to another.



(a)



FAD⁺ is another energetic molecule that can transport 2 electrons to other acceptors.

Lipoate or lipoic acid has two thiol groups that can undergo reversible oxidation to a disulfide bond. Because of its capacity to undergo oxidation-reduction reactions, lipoate can serve both as an electron hydrogen carrier and as an acyl carrier. It contains an amide linkage with a lysine molecule: the lipoyllysyl moiety is the prosthetic group of dihydrolipoyletransacetylase (E2 of the PDH complex).

The lipoyl group occurs in oxidised (disulfide) and reduced (dithiol) forms and acts as a carrier of both hydrogen and an acetyl (or other acyl) group. The five-reaction sequence of pyruvate dehydrogenase complex is an example of substrate channeling. The intermediates of the multistep sequence never leave the complex, the local concentration of substrates is kept very high and channeling also prevents the reaction of the activated acetyl group by other enzymes

COENZYMES OR COFACTORS

A cofactor is a non-protein chemical compound or metallic ion that is required for an enzyme's activity. Cofactors can be considered "helper molecules" that assist in biochemical transformations. Cofactors can be subclassified as either inorganic ions or complex organic molecules called coenzymes, the latter of which is mostly derived from vitamins and other organic essential nutrients in small amounts. A coenzyme that is tightly or even covalently bound is termed a prosthetic group.

TABLE 6-2 Some Coenzymes That Serve as Transient Carriers of Specific Atoms or Functional Groups

Coenzyme	Examples of chemical groups transferred	Dietary precursor in mammals
Biocytin	CO ₂	Biotin
Coenzyme A	Acyl groups	Pantothenic acid and other compounds
5'-Deoxyadenosylcobalamin (coenzyme B ₁₂)	H atoms and alkyl groups	Vitamin B ₁₂
Flavin adenine dinucleotide	Electrons	Riboflavin (vitamin B ₂)
Lipoate	Electrons and acyl groups	Not required in diet
Nicotinamide adenine dinucleotide	Hydride ion (H ⁻)	Nicotinic acid (niacin)
Pyridoxal phosphate	Amino groups	Pyridoxine (vitamin B ₆)
Tetrahydrofolate	One-carbon groups	Folate
Thiamine pyrophosphate	Aldehydes	Thiamine (vitamin B ₁)

The five-reaction sequence of pyruvate dehydrogenase complex is an example of substrate channeling. The intermediates of the multistep sequence never leave the complex, the local concentration of substrates is kept very high and channeling also prevents the reaction of the activated acetyl group by other enzymes.

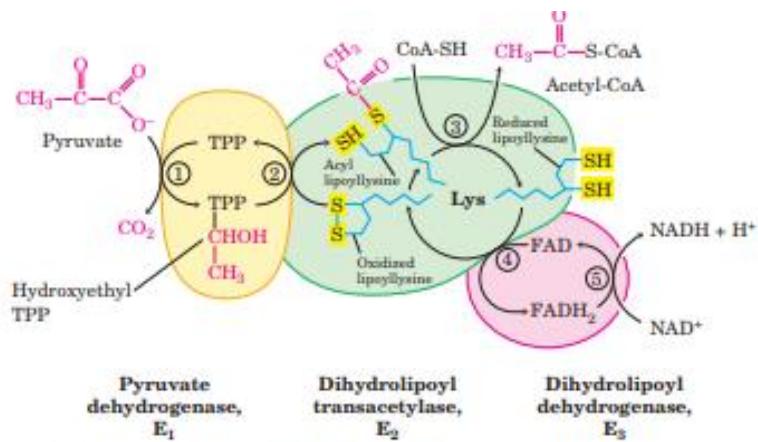


FIGURE 16–6 Oxidative decarboxylation of pyruvate to acetyl-CoA by the PDH complex. The fate of pyruvate is traced in red. In step ① pyruvate reacts with the bound thiamine pyrophosphate (TPP) of pyruvate dehydrogenase (E_1), undergoing decarboxylation to the hydroxyethyl derivative (see Fig. 14–13). Pyruvate dehydrogenase also carries out step ②, the transfer of two electrons and the acetyl group from TPP to the oxidized form of the lipoyllysyl group of the core enzyme, dihydrolipoyl transacetylase (E_2), to form the acetyl thioester of the reduced lipoyl group. Step ③ is a transesterification in which the

—SH group of CoA replaces the —SH group of E_2 to yield acetyl-CoA and the fully reduced (dithiol) form of the lipoyl group. In step ④ dihydrolipoyl dehydrogenase (E_3) promotes transfer of two hydrogen atoms from the reduced lipoyl groups of E_2 to the FAD prosthetic group of E_3 , restoring the oxidized form of the lipoyllysyl group of E_2 . In step ⑤ the reduced $FADH_2$ of E_3 transfers a hydride ion to NAD^+ , forming $NADH + H^+$. The enzyme complex is now ready for another catalytic cycle. (Subunit colors correspond to those in Fig. 16–5b.)

In step one, C-1 of pyruvate is released as CO_2 , and C-2, which in pyruvate has the oxidation state of an aldehyde, is attached to TPP as a hydroxyethyl group. This first step is the slowest and therefore limits the rate of the overall reaction. It is also the point at which the PDH complex exercises its substrate specificity. In step two the hydroxyethyl group is oxidised to the level of a carboxylic acid (acetate). The two electrons removed in this reaction reduce the disulfide bond of a lipoyl group on E_2 to two thiol groups. The acetyl moiety produced in this oxidation-reduction reaction is first esterified to one of the lipoyl thiol groups, then trans-esterified to CoA to form acetyl-CoA (step three). The remaining reactions catalysed by the PDH complex are electron transfers necessary to regenerate the oxidised form of the lipoyl group of E_2 to prepare the enzyme complex for another round of oxidation.

The electrons removed from the hydroxyethyl group derived from pyruvate pass through FAD to NAD^+ .

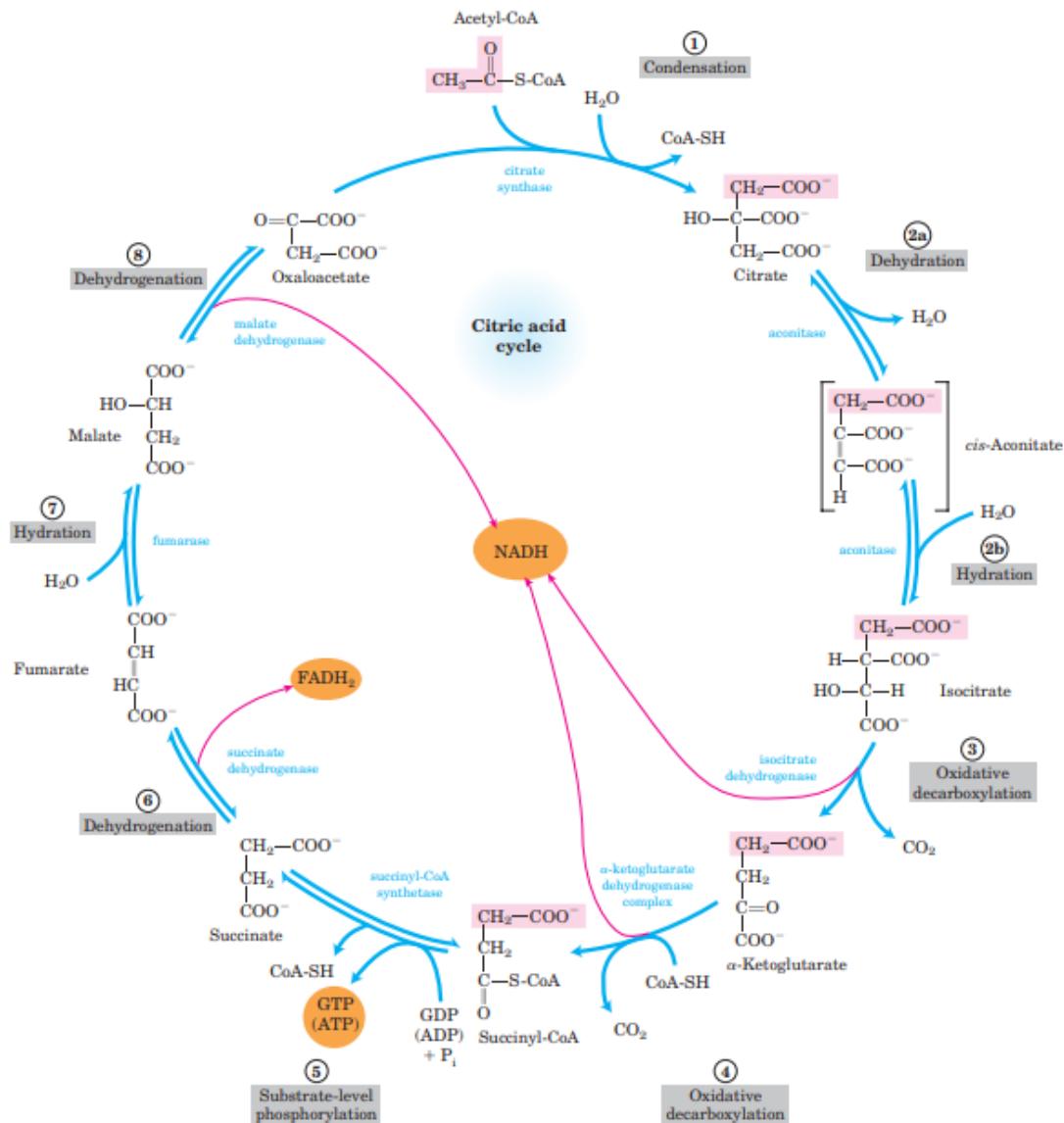


FIGURE 16-7 Reactions of the citric acid cycle. The carbon atoms shaded in pink are those derived from the acetate of acetyl-CoA in the first turn of the cycle; these are not the carbons released as CO₂ in the first turn. Note that in succinate and fumarate, the two-carbon group derived from acetate can no longer be specifically denoted; because succinate and fumarate are symmetric molecules, C-1 and C-2 are indistinguishable from C-4 and C-3. The number beside each

reaction step corresponds to a numbered heading on pages 608–612. The red arrows show where energy is conserved by electron transfer to FAD or NAD⁺, forming FADH₂ or NADH + H⁺. Steps ①, ③, and ④ are essentially irreversible in the cell; all other steps are reversible. The product of step ⑤ may be either ATP or GTP, depending on which succinyl-CoA synthetase isozyme is the catalyst.

After obtaining acetyl coenzyme A, we are ready to enter the citric acid cycle. It's a nearly universal central catabolic pathway in which compounds derived from the breakdown of carbohydrates, fats, and proteins are oxidised to carbon dioxide. As mentioned before, most of the energy of oxidation is temporarily held in the electron carriers FADH₂ and NADH. The citric acid cycle is amphibolic, serving in both catabolism and anabolism; cycle intermediates can be drawn off and used as the starting material for a variety of biosynthetic products. Acetyl-CoA enters the citric acid cycle through its condensation with oxaloacetate to form citrate. In eight sequential reactions the TCA converts citrate to oxaloacetate and releases two molecules of carbon dioxide. This pathway is cyclic: for each oxaloacetate consumed in the path, one is produced. For each acetyl CoA oxidised, the energy gain consists of three molecules of NADH, one FADH₂ and one nucleoside triphosphate (ATP or GTP).

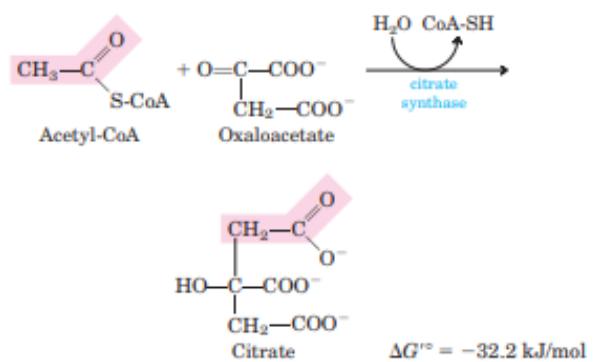
To begin a turn of the cycle, acetyl-CoA donates its acetyl group to the four-carbon compound oxaloacetate to form the six-carbon citrate (citrate synthase). Citrate is then transformed into

isocitrate (aconitase), also a six-carbon molecule, which is dehydrogenated with loss of CO₂ to yield the five-carbon compound α-ketoglutarate (also called oxoglutarate). α-ketoglutarate undergoes loss of a second molecule of CO₂ and ultimately yields the four-carbon compound succinate. Succinate is then enzymatically converted in three steps into the four-carbon oxaloacetate, which is then ready to react with another molecule of acetyl-CoA.

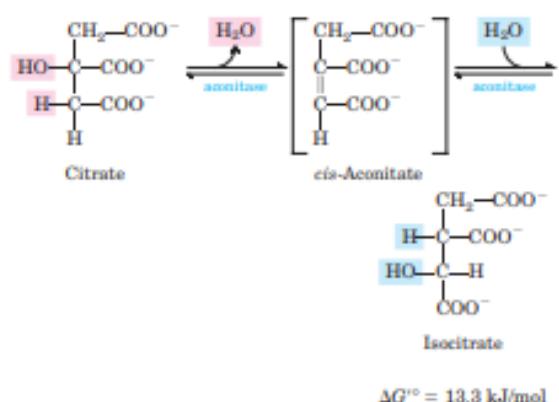
In each turn of the cycle, one acetyl group (two carbons) enters as acetyl-CoA and two molecules of CO₂ leave. One molecule of oxaloacetate is used to form citrate and one molecule of oxaloacetate is regenerated. No net removal of oxaloacetate occurs; one molecule of oxaloacetate can theoretically bring about oxidation of an infinite number of acetyl groups, and, in fact, oxaloacetate is present in cells in very low concentrations.

We now analyse the cycle step by step:

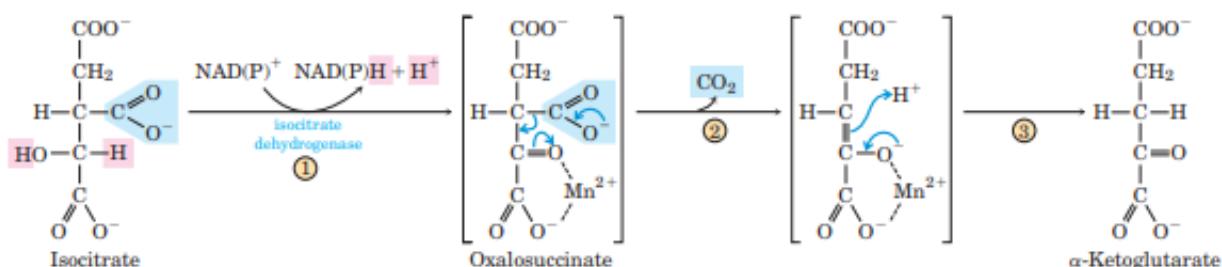
the first reaction of the cycle is the condensation of acetyl-CoA with oxaloacetate to form citrate, catalysed by citrate synthase. In this reaction the methyl carbon of the acetyl group is joined to the carbonyl group (C-2) of oxaloacetate. Citroyl-CoA is a transient intermediate formed on the active site of the enzyme. It rapidly undergoes hydrolysis to free CoA and citrate, which are released from the active site.



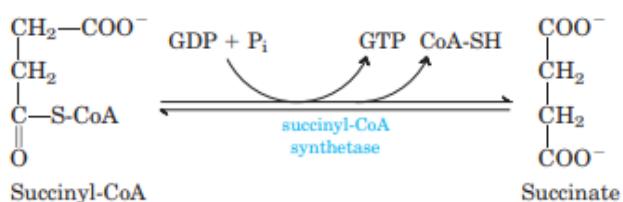
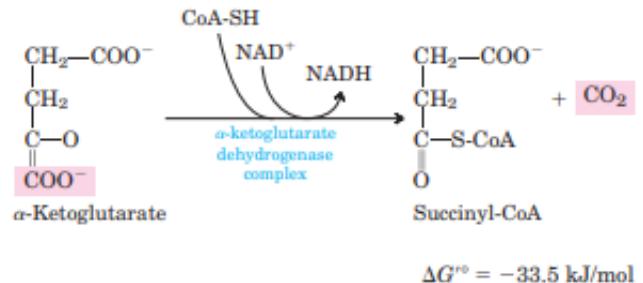
Step two is formation of isocitrate via cis-aconitate: the enzyme aconitase (aconitate hydratase) catalyses the reversible transformation of citrate to isocitrate, through the intermediary formation of the tricarboxylic acid cisaconitate. Aconitase can prove the reversible addition of water to the double bond of enzyme-bound cis-aconitate in two different ways, one leading to citrate and the other to isocitrate.



The third step is oxidation of isocitrate to α-ketoglutarate and CO₂: isocitrate dehydrogenase catalyses oxidative decarboxylation of isocitrate to form α-ketoglutarate. Mn²⁺ in the active site interacts with the carbonyl group of the intermediate oxalosuccinate, which is formed temporarily but does not leave the binding site until decarboxylation converts it to α-ketoglutarate. The cation also stabilises the enol formed by decarboxylation.

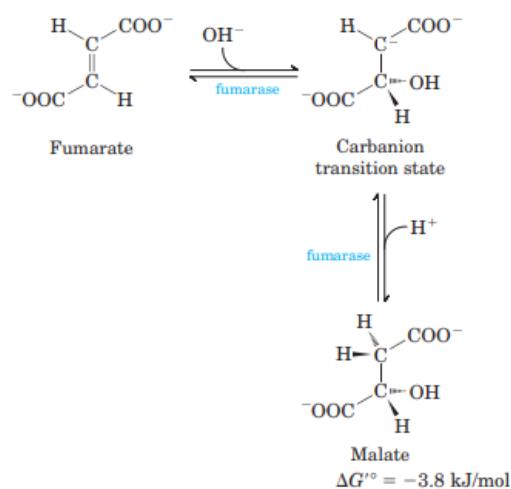
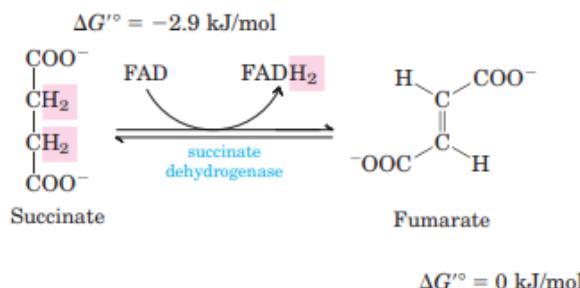


Reaction four is the oxidation of α -ketoglutarate to succinyl coenzyme A and CO_2 : this is another oxidative decarboxylation, in which α -ketoglutarate is converted to succinyl-CoA and CO_2 by the action of the α -ketoglutarate dehydrogenase complex. NAD^+ serves as electron acceptor and CoA as the carrier of the succinyl group. The energy of oxidation of α -ketoglutarate is conserved in the formation of the thioester bond of succinyl-CoA



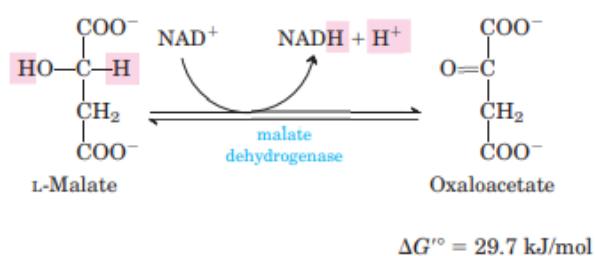
Step 5 is the conversion of succinyl coenzyme A to succinate: succinyl-CoA, like acetyl-CoA, has a thioester bond with a strongly negative standard free energy of hydrolysis. In this step of the citric acid cycle, energy released in the breakage of this bond is used to drive the synthesis of a phosphoanhydride bond in either GTP or ATP. The enzyme that catalyses this reversible reaction is called succinyl-CoA synthetase or succinic thiokinase; both names indicate the participation of a nucleoside triphosphate in the reaction.

The **sixth reaction** is oxidation of succinate to fumarate by the flavoprotein succinate dehydrogenase. Succinate dehydrogenase contains three different iron-sulfur clusters and one molecule of covalently bound FAD



Next, we have the hydration of fumarate (7) to L-malate: this reversible hydration is catalysed by fumarase (fumarate hydratase). The transition state in this reaction is a carbanion.

The **final step** is oxidation of malate to oxaloacetate: in this last reaction, L-malate dehydrogenase catalyses the oxidation of L-malate to oxaloacetate. The equilibrium of this reaction lies far to the left under standard thermodynamic conditions, but in intact cells oxaloacetate is continually removed by the highly exergonic citrate synthase reaction. This keeps the concentration of oxaloacetate in the cell extremely low pulling the malate dehydrogenase reaction toward the formation of oxaloacetate.



THE CITRIC ACID CYCLE

The citric acid cycle is amphibolic, serving in both catabolism and anabolism; cycle intermediates can be drawn off and used as the starting material for a variety of biosynthetic products. As intermediates of the citric acid cycle are removed to serve as biosynthetic precursors, they are replenished by anaplerotic reactions: under normal circumstances, the reactions by which cycle intermediates are siphoned off into other pathways and those by which they are replenished are in dynamic balance, so that the concentrations of the citric acid cycle intermediates remain almost constant.

The most important anaplerotic reaction in mammalian liver and kidney is the reversible carboxylation of pyruvate by CO₂ to form oxaloacetate, catalysed by pyruvate carboxylase. When the citric acid cycle is deficient in oxaloacetate or any other intermediates, pyruvate is carboxylated to produce more oxaloacetate.

The enzymatic addition of a carboxyl group to pyruvate requires energy, which is supplied by ATP: the free energy required to attach a carboxyl group to pyruvate is about equal to the free energy available from ATP. Pyruvate carboxylase is a regulatory enzyme and is virtually inactive in the absence of acetyl-CoA, its positive allosteric modulator. Whenever acetyl-CoA, the fuel for the citric acid cycle, is present in excess, it stimulates the pyruvate carboxylase reaction to produce more oxaloacetate, enabling the cycle to use more acetyl-CoA in the citrate synthase reaction.

The PDH complex is allosterically inhibited when ATP, NADH, fatty acids and acetyl coenzyme A ratios are high, indicating an energy-sufficient metabolic state. When these ratios decrease, allosteric activation of pyruvate oxidation results.

The flow of metabolites through the citric acid cycle is under stringent regulation. Three factors govern the rate of flux through the cycle: substrate availability, inhibition by accumulating products, and allosteric feedback inhibition of the enzymes that catalyse early steps in the cycle.

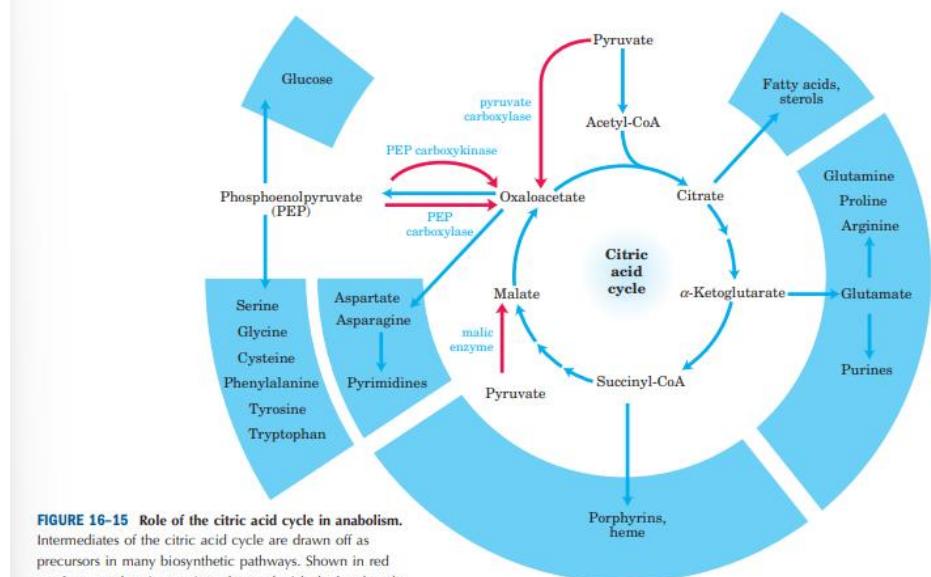
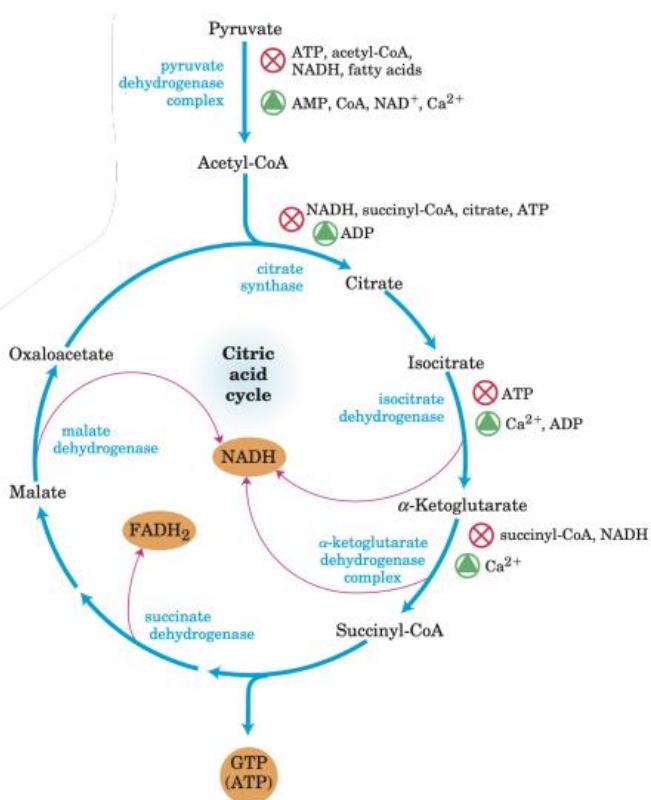
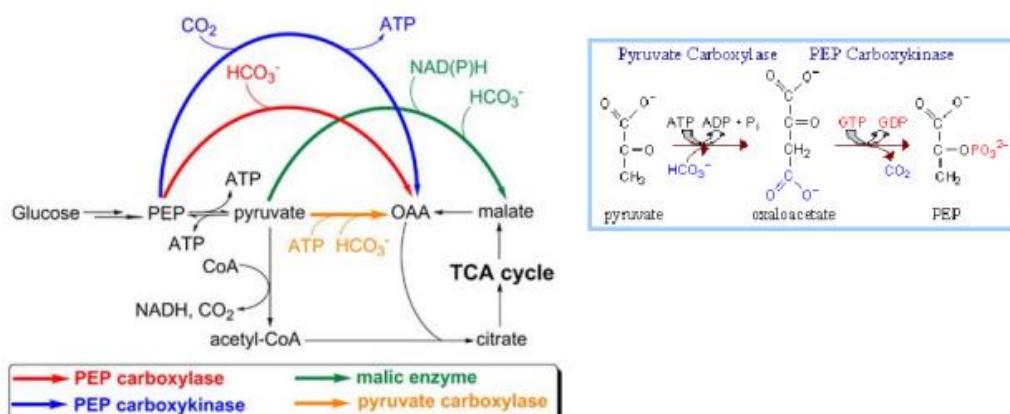


FIGURE 16–15 Role of the citric acid cycle in anabolism. Intermediates of the citric acid cycle are drawn off as precursors in many biosynthetic pathways. Shown in red are four anaplerotic reactions that replenish depleted cycle intermediates (see Table 16–2).



ANAPLEROTIC REACTIONS



PEP carboxylase (higher plants, yeasts, bacteria; PEP carboxykinase (heart, muscle);
Malic enzyme (eukaryotes, prokaryotes); pyruvate carboxylase (Liver, kidney)

Anaplerosis, from Ancient Greek ἀνά (aná, “up”) + πληρώ (plérōō, “to fill”) → to fill up.

As intermediates of the citric acid cycle are removed to serve as biosynthetic precursors, they are replenished by anaplerotic reactions.

We already see some of these reaction during nucleogenesis.

11-TCA cycle regulation and fatty acid degradation

Regulation of pyruvate dehydrogenase

We have 2 levels of regulation:

-Allosteric regulation:

Pyruvate dehydrogenase is regulated by different allosteric inhibitors and activators; again, the concept is that when cells have a lot of energy the pyruvate dehydrogenase is inhibited, and so ATP, Acetyl-coA, NADH and fatty acids are allosteric inhibitors of dehydrogenase.

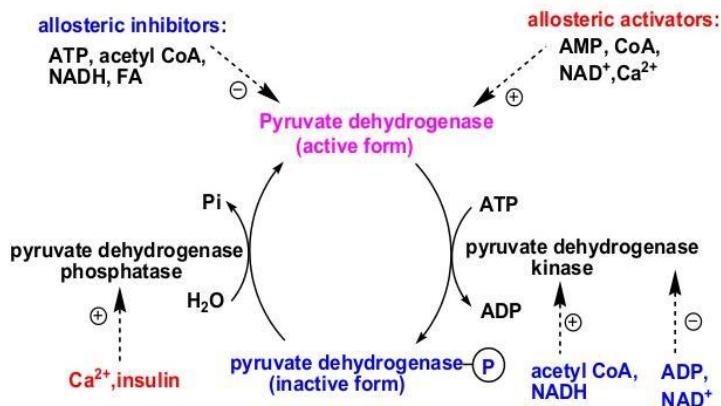
On the contrary, when cells have low amount of energy, pyruvate dehydrogenase must be activated and in fact, AMP, coA, oxidized NAD, and calcium are allosteric activators of pyruvate dehydrogenase. (in particular calcium is an activator in muscle cells)

-Regulation by phosphorylation

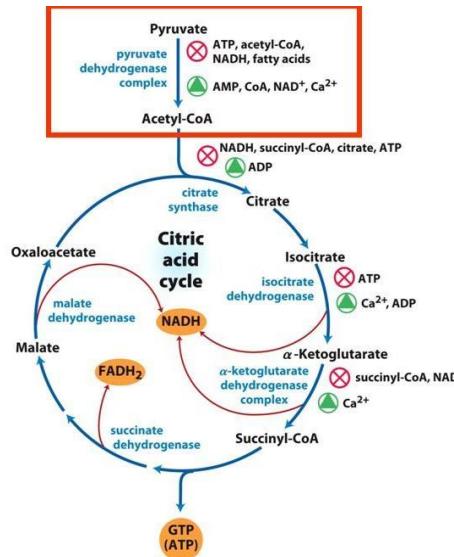
Pyruvate dehydrogenase is also regulated by phosphorylation: when pyruvate dehydrogenase is phosphorylated it is inactive, when it is not phosphorylated it is active. Only when pyruvate dehydrogenase is dephosphorylated it is sensitive to the allosteric enzymes

The enzyme that catalyzes the phosphorylation of pyruvate dehydrogenase is called pyruvate dehydrogenase kinase, which is an enzyme activated allosterically by acetyl-coA and NADH (these are the same molecules that are also allosteric inhibitors of pyruvate dehydrogenase) and inactivated by ADP and NAD+ (also more or less the same allosteric activators of pyruvate dehydrogenase).

The enzyme that dephosphorylates pyruvate dehydrogenase is called pyruvate dehydrogenase phosphatase, and it is activated by insulin.



Regulation of citric acid cycle



Acetyl-coA is committed to be completely oxidized in Krebs Cycle, or, if it accumulated, it is the substrate for the synthesis of fatty acids.

The reactions that are regulated in Krebs Cycle are the first reactions (because they are exergonic and irreversible). The other reactions are reversible, so they can be used during oxidative citric acid cycle (to form NADH and FADH₂), but when cells have more energy, the intermediates of Krebs cycle can be used for the synthesis of amino acids, purines...

So, the Krebs cycle is regulated in its oxidative part (in which we have the decarboxylation of Acetyl-coA), but after the other intermediates can also be used for the anabolic pathway.

Reactions that are regulated:

- >condensation of oxalacetate with Acetyl-coA
- >oxidation of isocitrate into alpha-ketoglutarate
- >oxidation of alpha-ketoglutarate into Succinyl-coA

Citric acid cycle is regulated depending on the need of energy, so ATP and NADH are the most important regulators

Three factors govern the rate of flux through the cycle:

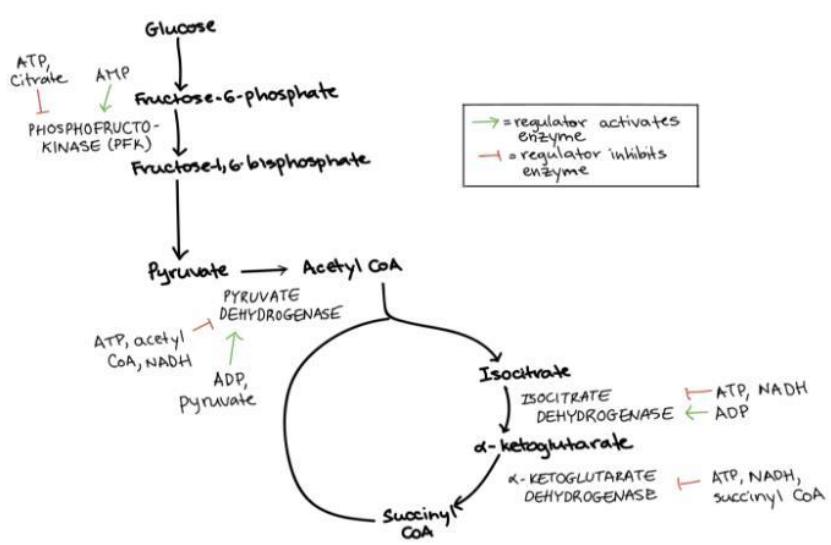
- substrate availability,
- inhibition by accumulating products,
- allosteric feedback inhibition of the enzymes that catalyze early steps in the cycle.

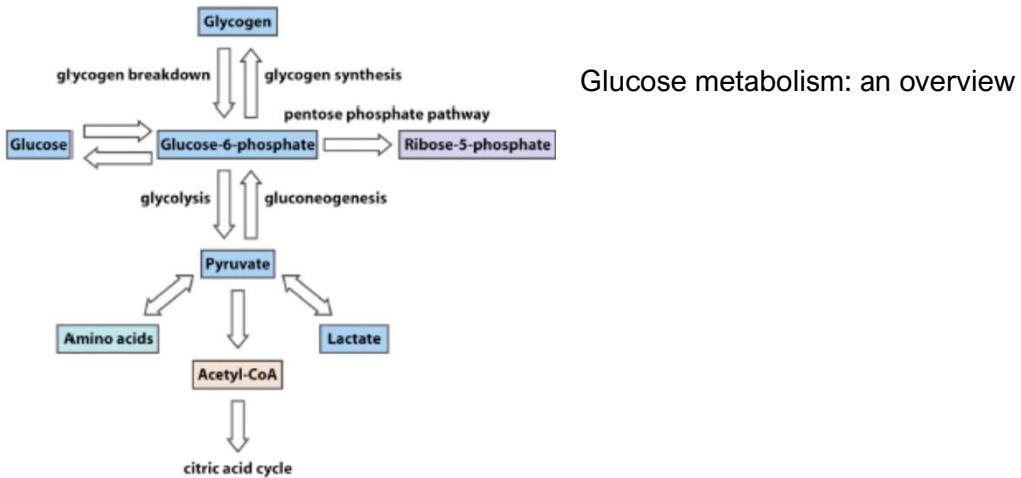
Integrated regulation of glycolysis and citric acid cycle

In this scheme, we have the most important allosteric regulators of glycolysis, of the oxidation of pyruvate and of the citric acid cycle. We want to stress the concept that high energetic level inhibits glycolysis and the aerobic fate of pyruvate; low energetic levels (represented by the presence of AMP or ADP) are activators of glycolysis and, after, of the aerobic fate of pyruvate.

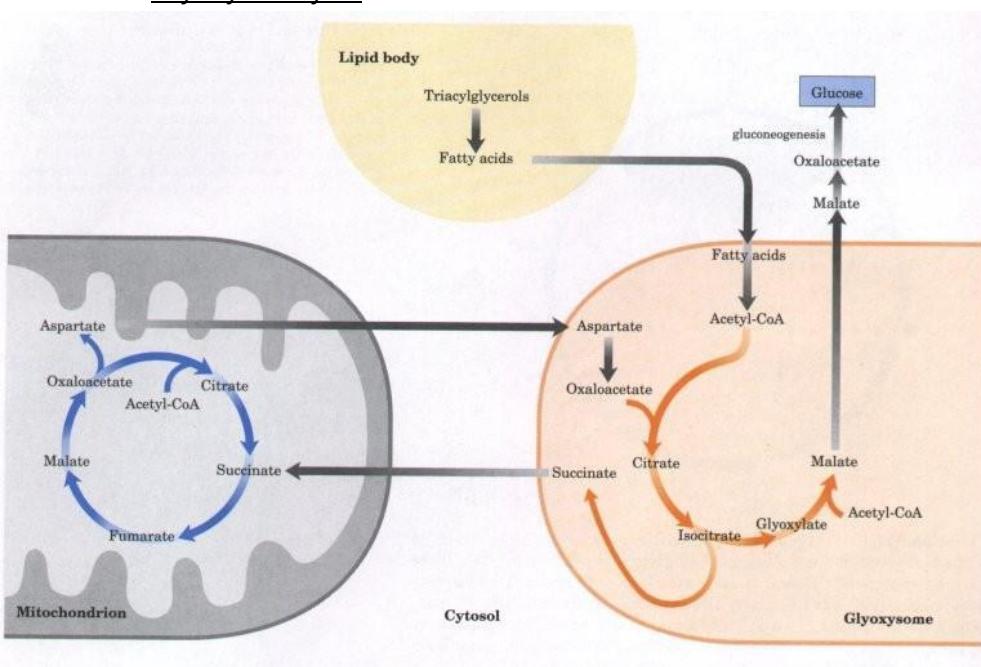
Most important regulated reactions (because they are sensitive to the energetic levels) are catalyzed by:

- >PFK 1
- >Pyruvate dehydrogenase
- >isocitrate dehydrogenase
- >alpha-ketoglutarate dehydrogenase





Glyoxylate cycle



This cycle is called glyoxylate cycle because one of the intermediates is a molecule called glyoxylate. It is a cycle which is not present in animal cells, whereas it is active in the germinating seeds of some plants and in certain microorganisms that can live on acetate as the sole carbon source.

Cells that perform this cycle can use the oxidation of fatty acids to synthesize glucose. (Animals cannot)

In fact, these cells are able to use Acetyl-coA and substrates that produce Acetyl-coA (like fatty acids) for synthesizing glucose.

This is very important in germinating seeds, because they are very rich in fatty acids, and when they germinate, they can synthesize all the molecules for producing the first leaves to start the photosynthesis and synthesize glucose through it. The production of glucose is important not only for the energetic level, but also because we have seen that from glucose, we can synthesize different intermediates for other metabolic pathways.

How it works:

In seeds we have several lipid bodies, whose triacylglycerols are degraded into fatty acids, which are then further degraded to form Acetyl-coA. (This pathway is called oxidation of fatty acids)

Acetyl-coA in this case enters in specific organelles called glyoxysomes, and then it starts reactions that are very similar to the TCA cycle. (The enzymes present in glyoxysomes are the same of mitochondria)

GIULIA= The glyoxylate cycle was discovered by Krebs and is present in germinating seeds of some plants and some microorganisms, which can degrade molecules that are not carbohydrates to form acetyl coA, from which they use the glyoxylate

cycle to produce glucose. Animal cells are not capable of doing this. Germinating seeds can use this cycle to start their growth until the plant is able to perform photosynthesis. It's similar to the Krebs' cycle, but it lacks the oxidative reactions. It occurs in the glyoxysome, an organelle. In the glyoxylate cycle, acetyl-CoA condenses with oxaloacetate to form citrate, and citrate is converted to isocitrate, exactly as in the citric acid cycle. The next step, however, is not the breakdown of isocitrate by isocitrate dehydrogenase but the cleavage of isocitrate by isocitrate lyase, forming succinate and glyoxylate. The glyoxylate then condenses with a second molecule of acetyl-CoA to yield malate, in a reaction catalysed by malate synthase. The malate is subsequently oxidised to oxaloacetate, which can condense with another molecule of acetyl-CoA to start another turn of the cycle. The succinate may be converted through fumarate and malate into oxaloacetate, which can then be converted to PEP by PEP carboxykinase, and thus to glucose by gluconeogenesis. The same intermediates of glycolysis and the citric acid cycle that activate isocitrate dehydrogenase are allosteric inhibitors of isocitrate lyase. When glycolytic and citric acid cycle intermediates are low in concentration, isocitrate dehydrogenase is inactivated, the inhibition of isocitrate lyase is relieved, and isocitrate flows into the glyoxylate pathway, to be used in the biosynthesis of carbohydrates, amino acids, and other cellular components.

In fact, we have the condensation of Acetyl-coA with Oxaloacetate to form Citrate. After, citrate is isomerized to form isocitrate, which is then split in 2 molecules: glyoxylate and Succinate (this reaction is different to TCA cycle)

-Glyoxylate is a molecule that can be condensated with another molecule of Acetyl-coA (only in this organelle), and from this condensation we have the formation of malate.

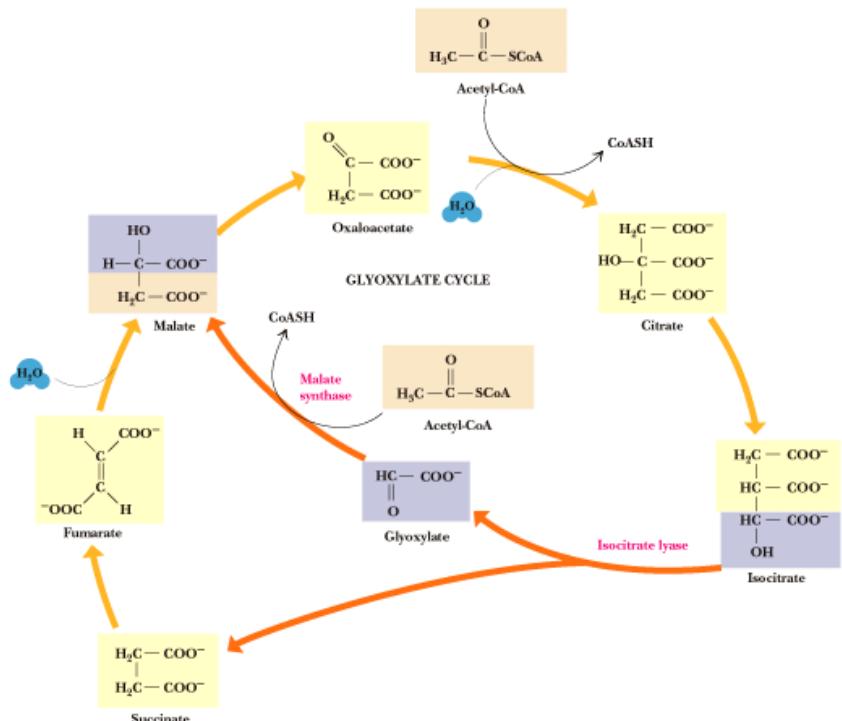
Malate, in this case, is transported is transport out from the glyoxysome and it is oxidized to form oxalacetate.

From oxalacetate (intermediate of gluconeogenesis) cells are able to synthetize glucose through gluconeogenesis.

In this way, with the condensation of 2 molecules of Acetyl-coA deriving from fatty acids oxidation, in glyoxysome we have the ability to synthetize a molecule with 4 carbons that is malate.

Here we don't have the oxidative reactions (like TCA), because glyoxylate cycle is an anabolic pathway.

-Succinate is transported out and it can enter inside mitochondria, where there are the enzymes that can perform the TCA cycle, and at the end we have oxalacetate as a product, which can be recycled for a new cycle. But oxalacetate cannot exit mitochondria, because there is no carrier for it, so it is aminated to aspartate. Aspartate can then be transported across mitochondria and enter again in glyoxysome, where it is deaminated to form oxalacetate again.



Fatty acid catabolism

Cells can obtain fatty acid fuels from three sources:

- Fats consumed in the diet,
- Fats stored in cells as lipid droplets,
- Fats synthesized in one organ (for export to another).

Fatty acids and lipids are the most important stores of energy, because they are highly hydrophobic and so they can be concentrated in lipid droplets, in which water is eliminated > in this way we have a lot of molecules that can be oxidized in a little volume.

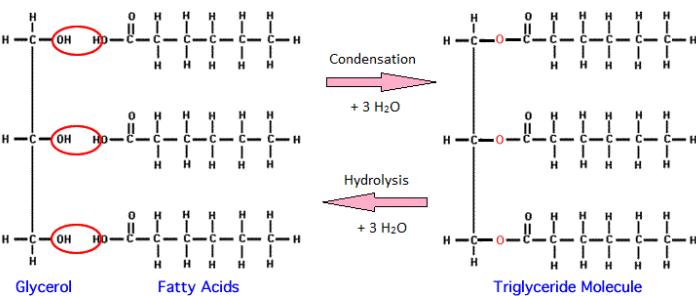
We know that another energetic storage is glycogen, but it is very hydrated; and the weight of glycogen must be 3 times the weight of lipids to give the same amount of energy.

This is the reason why glycogen is only a daily store.

The most important lipids used as store are triacylglycerols, which are molecules formed with a

molecule of glycerol, an alcohol with 3 hydroxyl groups, which are condensed and esterified with 3 chains of fatty acids. The fatty acids can be saturated fatty acids, or unsaturated fatty acids, depending on which our cells are able to synthesize.

When we have a saturated fatty acid, we obtain the most reduced molecules. When we have unsaturated fatty acids, we have molecules that are less reduced,



so we can obtain less energy (but still a lot). Triacylglycerols provide more than half the energy requirements of some organs, particularly the liver, heart, and resting skeletal muscle.

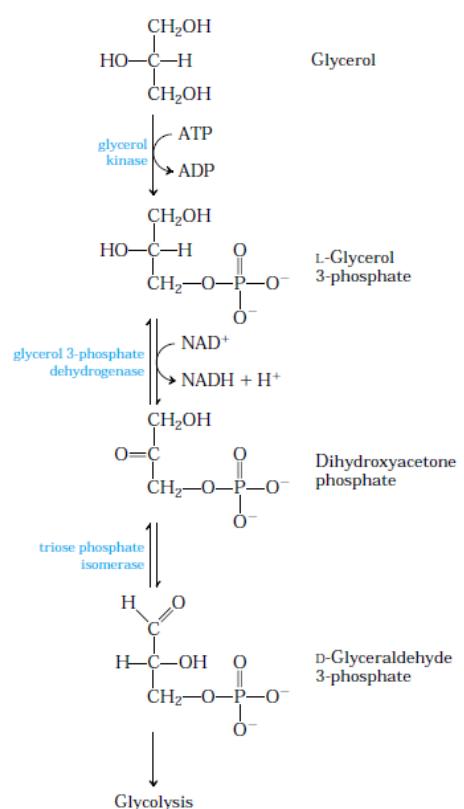
Stored triacylglycerols are virtually the sole source of energy in hibernating animals and migrating birds. (because in this way they can store a lot of energy in little weight). Vascular plants mobilize fats stored in seeds during germination, but do not otherwise depend on fats for energy.

The first step of hydrolysis of triacylglycerols occurs in cytosol with the formation of one molecule of glycerol and three molecules of fatty acids (triacylglycerols degraded to produce energy)

About 95% of the biologically available energy of triacylglycerols resides in their three long-chain fatty acids.

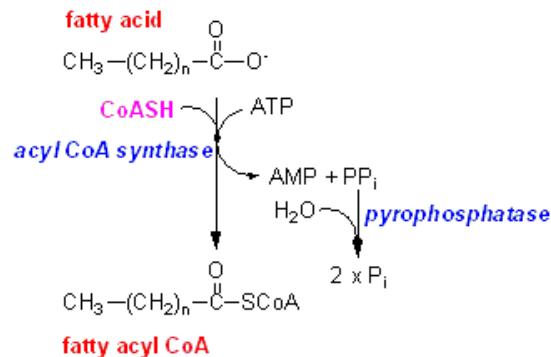
Only 5% is contributed by the glycerol moiety. The glycerol released by lipase action is phosphorylated by glycerol kinase to glycerol 3-phosphate, this usually occurs in liver. After it is oxidized to dihydroxyacetone phosphate (intermediate of glycolysis or of gluconeogenesis). (Glycerol is the only part of triacylglycerols that can be used for gluconeogenesis)

The glycolytic enzyme triose phosphate isomerase converts this compound to glyceraldehyde 3-phosphate, which is oxidized via glycolysis.



After, the 3 fatty acids have to be degraded to form Acetyl-coA, and this occurs in cytosol.

In fact, fatty acids must be transported inside mitochondria to be oxidized. But they are not transported as free fatty acids, they are activated to form fatty acyl coenzyme A. This reaction is catalyzed by the enzyme acyl CoA synthase, which uses as substrates a fatty acid, free coenzyme A and ATP, because the condensation of coenzyme A with fatty acids is an endergonic reaction.



In this reaction we have also the formation of pyrophosphate: as we know, in reactions of hydrolysis of nucleotides (ATP, GTP, UTP), pyrophosphate is immediately hydrolyzed to form 2 molecules of phosphate, and this is a very exergonic reaction.

Now that our fatty acid is activated it can be transported inside mitochondria.

The carnitine shuttle

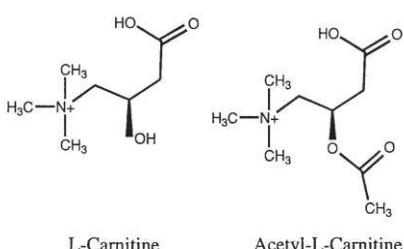


Figure 1 Chemical structures of carnitine and acetyl-L-carnitine

The transport of fatty acids inside mitochondria is regulated: fatty acids are hydrophobic molecules, so, when they are short (4/6 carbons), they can pass across the membranes of mitochondria by diffusion.

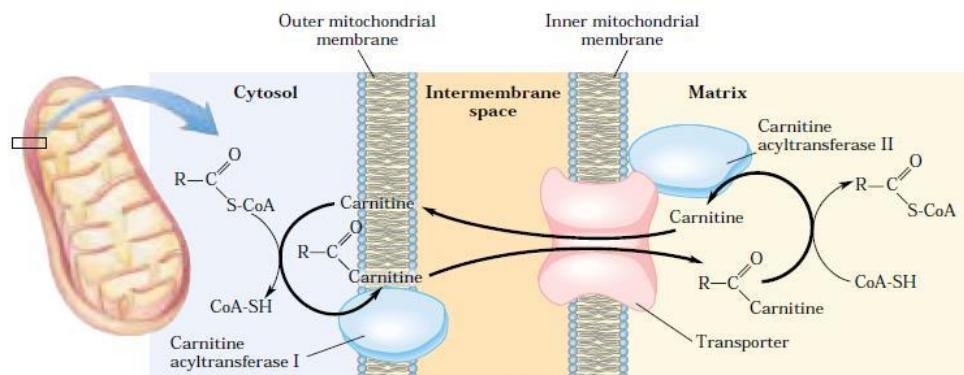
But, when they are long molecules (often: 14-16-18 carbons), they need to be transported across the 2 membranes of mitochondria by a specific shuttle called carnitine shuttle, because the molecule involved in the shuttle is L-carnitine. L-carnitine is a molecule with an hydroxylic group, which can react with Acetyl-coA to form Acetyl-L-carnitine.

This reaction is catalyzed by Carnitine acyltransferase I, which is an enzyme present in the outer mitochondrial membrane.

Then Acetyl-L-carnitine can be diffused across the outer mitochondrial membrane, but, for the transport of Acetyl-L-carnitine inside the matrix, cells need a specific transporter in the inner mitochondrial matrix (very impermeable, only molecules with a specific carrier can pass through it).

Therefore, the Acetyl-L-carnitine can enter inside mitochondria, where we have the second by Carnitine acyltransferase (II), which, inside the matrix, can catalyze the reverse reaction that we have seen in cytosol, so we have the formation of Acetyl-coA and of free carnitine, which can exit from the mitochondria. The transporter in this case is called antiporter: because for each molecule of Acetyl-L-carnitine that enters, there one of carnitine that exits.

Now we have Acetyl-coA inside the mitochondria.



Fatty acid catabolism

Fatty acid catabolism is strictly correlated with an aerobic metabolism, so, while glucose can be oxidized also in absence of oxygen, the oxidation of fatty acids requires an aerobic condition.

In fact, we will see that in the first stage of oxidation of fatty acids, also called beta oxidation, we don't have the production of ATP, but we have the production of FADH₂ and NADH, so it is very important that after we have an active respiratory chain to release the energy.

At the end of the oxidation of fatty acids we have the formation of molecules of Acetyl-coA, which, in an aerobic metabolism, is then oxidized in the citric acid cycle.

Mitochondrial oxidation of fatty acids takes place in three stages:

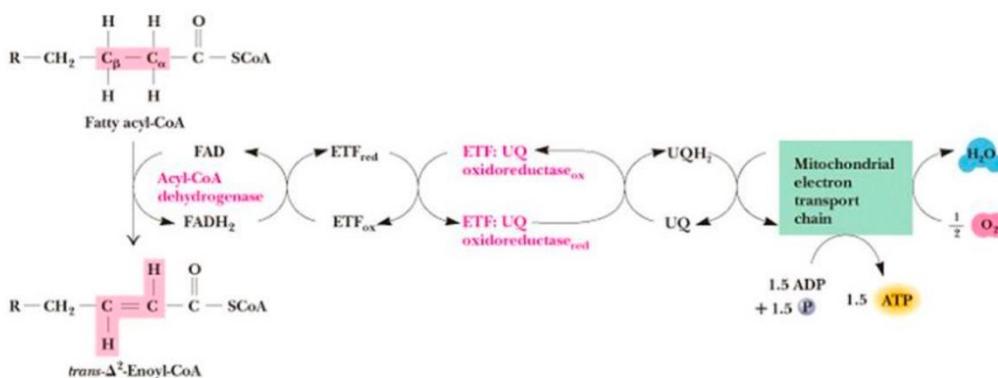
Stage 1: a long-chain fatty acid is oxidized to yield acetyl residues in the form of acetyl-CoA.

Oxidation occurs at C_b → called b-oxidation

Stage 2: the acetyl groups are oxidized to CO₂ via the citric acid cycle

Stage 3: electrons derived from the oxidations are delivered to the mitochondrial respiratory chain, providing the energy for ATP synthesis by oxidative phosphorylation.

STEP 1: OXIDATION OF C_a-C_b BOND



The first reaction is an oxidation between the carbon a and the carbon b.

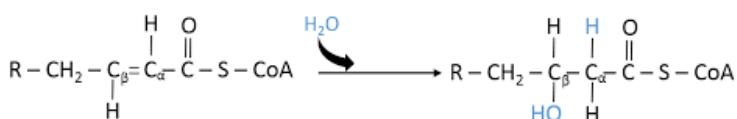
The reaction is catalyzed by the enzyme acyl-CoA dehydrogenase, which can oxidize the bond to form a double bond. So, there is the formation of a trans

saturated fatty acid: trans-Δ³-enoyl-CoA. The product of this reaction is FADH₂, because acyl-CoA dehydrogenase is an enzyme that has as coenzyme FAD.

There are three isozymes of acyl-CoA dehydrogenase, each specific for a range of fatty-acyl chain lengths.

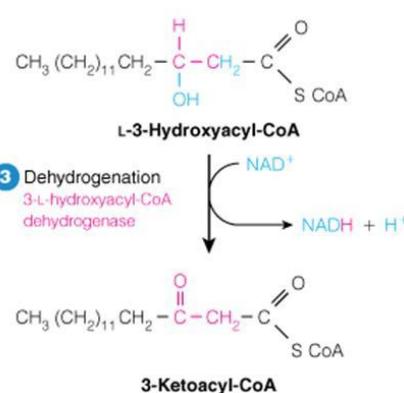
The electrons removed from the fatty acyl-CoA are transferred to FAD, that immediately donates its electrons to an electron carrier of the mitochondrial respiratory chain, the electron-transferring flavoprotein (ETF). This protein is also substrate of another protein, which is ETF: UQ oxidoreductase, which is present inside the inner mitochondrial membrane and can extract electrons from ETF to give them to UQ.

STEP 2 – HYDRATION OF ENOIL-CoA TO HYDROXYACIL-CoA



with cis enoil-CoA).

STEP 3 – OXIDATION OF HYDROXYACIL-CoA



Now the hydroxyacyl-CoA can be oxidized to form a ketone group at the level of the beta carbon, by an enzyme that is a dehydrogenase, which is absolutely specific for the L stereoisomer of hydroxyacyl-CoA. In this case the coenzyme involved is NAD, so we have the formation of a molecule of NADH. The NADH formed in the reaction donates its electrons to NADH dehydrogenase, an electron carrier of the respiratory chain. At the end we have 3-Ketoacyl-CoA. The presence of the ketone group induces the molecule to be better oxidized and the beta bond easily broken.

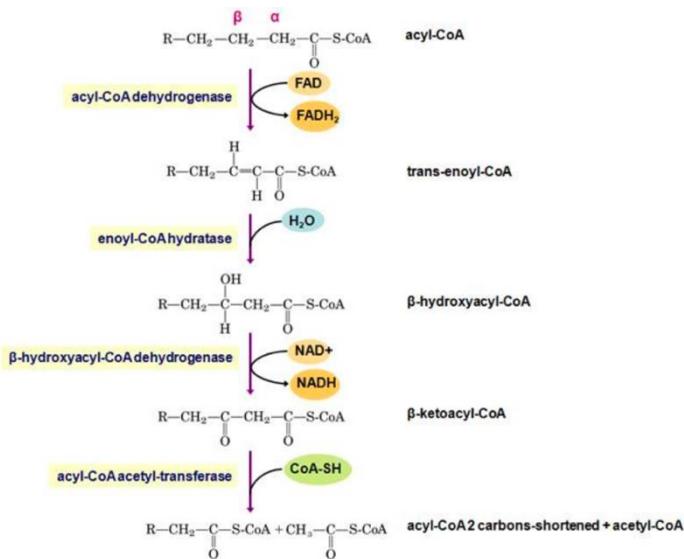
STEP 4 – SPLITTING OF KETOACYL-CoA TO ACETYL-CoA AND ACYL-CoA

The reaction of degradation of the beta bond is called thiolysis, catalyzed by the enzyme thiolase.

In this case we don't have a real hydrolysis (because we don't have the addition of a molecule of water), but coenzyme A can react with 3-Ketoacyl-CoA and the energy deriving from the break of the bond is used for the synthesis of a molecule of Acyl CoA, that is shortened by 2 carbons.

In this case the energy is conserved into the thioester bond of the acyl group. So, we have an activated fatty acid, which can be oxidized again with these 4 reactions, and a acetyl CoA, which can go in the Krebs cycle.

b- OXIDATION SUMMARY(or fatty acid oxidation)



Down we can see the stoichiometry of the complete degradation of a molecule of Palmitoyl-coA (16C) to form 8 (16/2) molecules of acetyl-CoA.

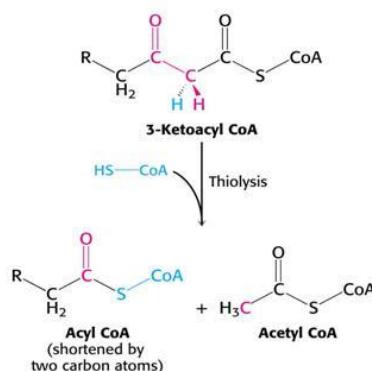
We can see that there is no production of ATP.

The most important reaction are four:

- 2 reactions of oxidation:

- First: formation of the molecule of FADH₂
- Third: formation of the molecule of NADH

- Hydrolysis



- Thiolysis: acyl CoA is shortened by two carbons, and we have formation of acetyl CoA.

When we have the oxidation of unsaturated fatty acids or polyunsaturated fatty acids, two additional enzymes, isomerase and reductase, are required for this reaction. The goal is to produce a *trans* double bond between C2 and C3.

Oxidation of unsaturated fatty acids

Most of the fatty acids in the triacylglycerols and phospholipids of animals and plants are unsaturated, having one or more double bonds. These bonds are in the *cis* configuration and cannot be acted upon by enoyl-CoA hydratase. Two auxiliary enzymes are needed for oxidation of the common unsaturated fatty acids: an isomerase and a reductase and, depending on the type of unsaturated fatty acids, one or both can be used. The goal is to obtain a *trans* unsaturated bond at position 2C-3C essential to go on β -oxidation

Oxidation of a monounsaturated fatty acid >

This is the oxidation of Oleoyl-coA, which is usually present in olive oil, and which is unsaturated between carbon 9 and 10. The first part of its oxidation occurs like a normal saturated fatty acid: so, we have the 3 steps of beta oxidation. But, at a certain point of the beta oxidation, we have the formation of an acyl coA in which we have a double bond in *cis* between carbon beta and gamma, which is not the substrate of the hydratase. SO, oxidation of oleic acid requires an additional enzyme, enoyl-CoA isomerase to

convert the *cis* isomer to a *trans* isomer, shifting the double bond from the position 3 and 4 to the position 2 and 3 (β bond).

In this way we skip the first oxidation (by acyl coA dehydrogenase) because we already have a double bond.

So in this case we have the production of a lower number of molecules of FADH₂

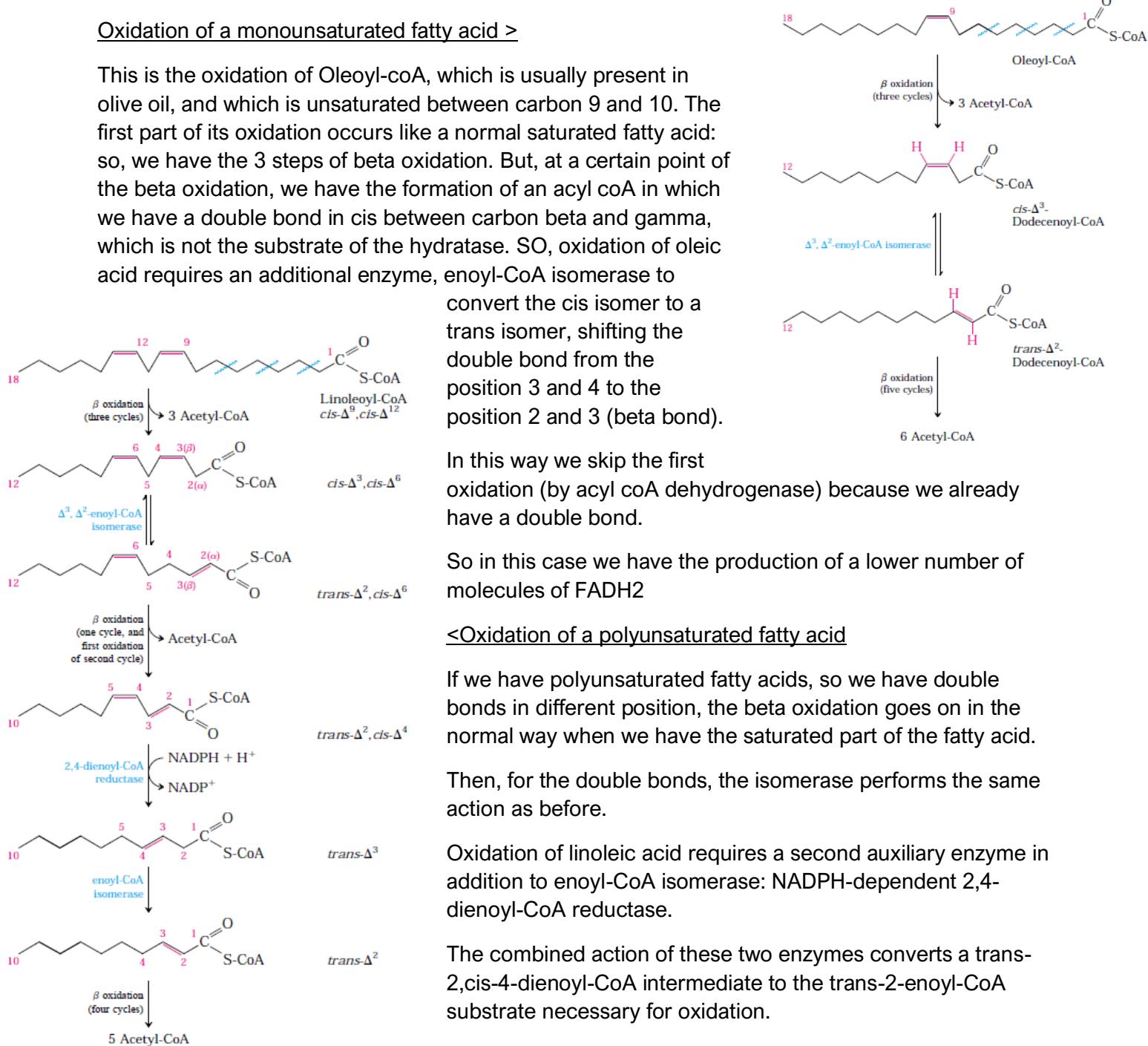
<Oxidation of a polyunsaturated fatty acid

If we have polyunsaturated fatty acids, so we have double bonds in different position, the beta oxidation goes on in the normal way when we have the saturated part of the fatty acid.

Then, for the double bonds, the isomerase performs the same action as before.

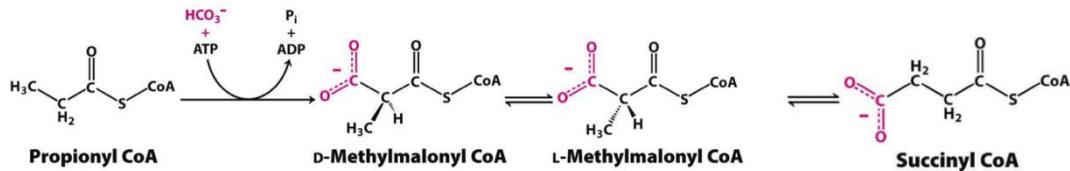
Oxidation of linoleic acid requires a second auxiliary enzyme in addition to enoyl-CoA isomerase: NADPH-dependent 2,4-dienoyl-CoA reductase.

The combined action of these two enzymes converts a *trans*-2,cis-4-dienoyl-CoA intermediate to the *trans*-2-enoyl-CoA substrate necessary for oxidation.



12-Ketone bodies

OXIDATION OF ODD NUMBER OF FATTY ACIDS

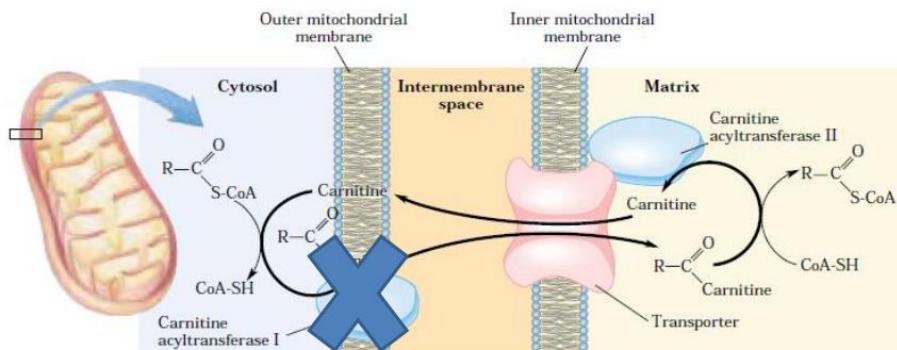


If cells oxidized fatty acids with an odd number of carbons, at the end there is the formation of a molecule of propionyl-CoA (3C). it is not an intermediate, so needs to be modified in a molecule that can enter in Krebs cycle. It is then rearranged to form succinyl-CoA. This reaction requires a carboxylation to form an intermediate called D-methyl malonyl-CoA, which is catalysed by an enzyme able to carboxyl the propionyl-CoA and rearrange the methyl malonyl CoA to form succinyl-CoA.

During the formation of the intermediates, we also have the formation of a radical, which is a very reactive molecule. So it is important that enzyme works properly in order to avoid the formation of radicals. Usually, this occurs and at the end of the reactions, also after the hydrolysis of a molecule of ATP, we obtain succinyl-CoA.

The oxidation of fatty acids with an odd number of carbon can produce energy, because succinyl-CoA is a molecule with an high energetic level, but require also energy for their metabolism.

REGULATION OF FATTY ACIDS OXIDATION



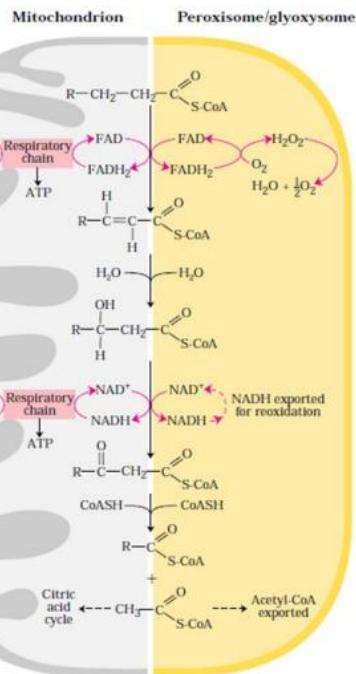
The regulation of fatty acids oxidation is very easy differently from the regulation of carbohydrates. In fact, the only regulated step is at the level of the transport of our fatty acid across the mitochondrial membrane.

When our fatty acid is inside the matrix, it is committed to go versus the oxidation and the enzyme of the β -oxidation are not particularly regulated, their activity depends on the availability of the substrate.

So the regulation of the entrance of the fatty acids inside mitochondria depend on the regulation of carnitine acyltransferase I (the first enzyme that transport them inside). It is inhibited by the first substrate (malonyl-CoA, the first molecule needed for synthesis) that indicates the activation of the synthesis of fatty acids.

It is a simple method for inhibiting two different and opposite metabolic pathway that occur in two different compartments (synthesis in cytosol, beta oxidation in matrix) and the activity of the anabolic pathway inhibits the activity of the catabolic one, blocking the entrance of the substrate.

ALTERNATIVE PATHWAYS OF FATTY ACIDS OXIDATION



The beta oxidation can occur in other organelles: in plant cells and animal cells occur peroxisomes and glyoxysome (only in plants and bacteria).

In glyoxysome the oxidation produces acetyl CoA, which can be used glyoxylates cycle to synthetase glucose. To form acetyl CoA there are reactions very similar to beta oxidation. The only difference is the first reaction: in mitochondria, it is produced FADH₂ that goes directly to respiration, while in peroxisomes and glyoxysome, FADH₂ is directly oxidized by O₂ to form H₂O₂. This is the reason for which peroxisome are called like that. The production of H₂O₂ is useful for molecules to kill microbes for example.

In peroxisomes, the fate acetyl CoA is for the synthesis of steroid lipids.

The oxidation of fatty acids is often used for: production of energy, glyoxylate cycle, synthesis of steroid and, in peroxisome, of H₂O₂ for the defence of our body.

KETONE BODIES

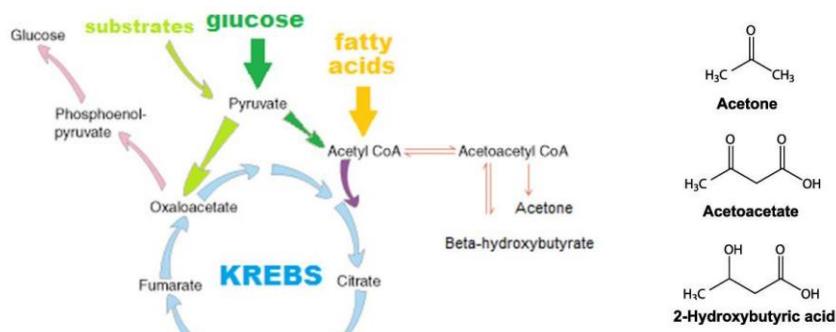
The oxidation of fatty acids may have particular fates when we are in fasting or starving state. This fate is specific in mammals cells for liver. Liver is the most important organ for the beta oxidation because in this way can produce energy for gluconeogenesis.

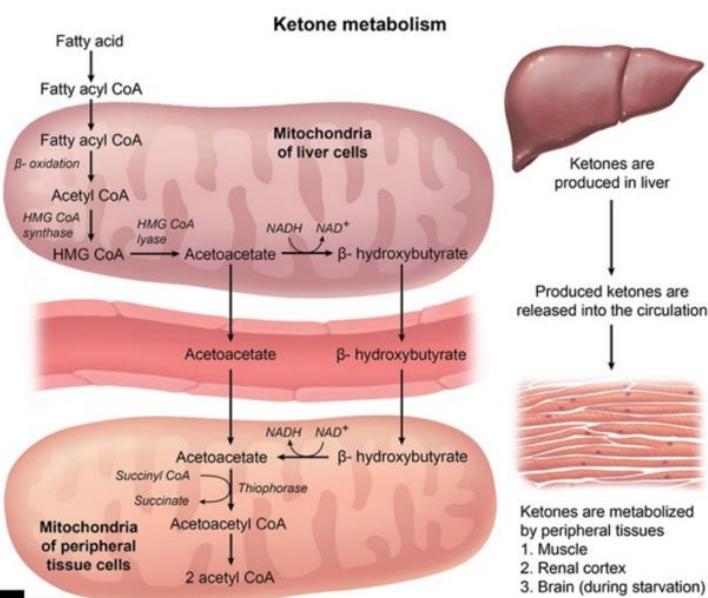
In specific conditions, the beta oxidation in liver is to fast the production of acetyl CoA which cannot enter entirely in Krebs cycle. Why? Because when we are in fasting or starving state, our liver is activated to synthesize glucose for the other organs. So oxaloacetate is shifted versus the glucose and it becomes limiting for the first reaction of Krebs cycle. We have a lot of degradation of fatty acids, a lot of formation of acetyl CoA, which enters in Krebs cycle starting from the first reaction. But oxaloacetate is limiting. Then the excess of acetyl CoA is condensed to form the so called **ketone bodies**, molecules with a ketone group. The ketone bodies produced in liver are: acetone, acetoacetate, and 2-hydroxybutyrate (not a real ketone, but derives from the excess of acetyl CoA).

What is the significance of their formation?

Ketone bodies can pass across the plasmatic membrane, can be released in blood and they are carbon sources for energy because they can be uptake by the other organs and modify for producing acetyl CoA.

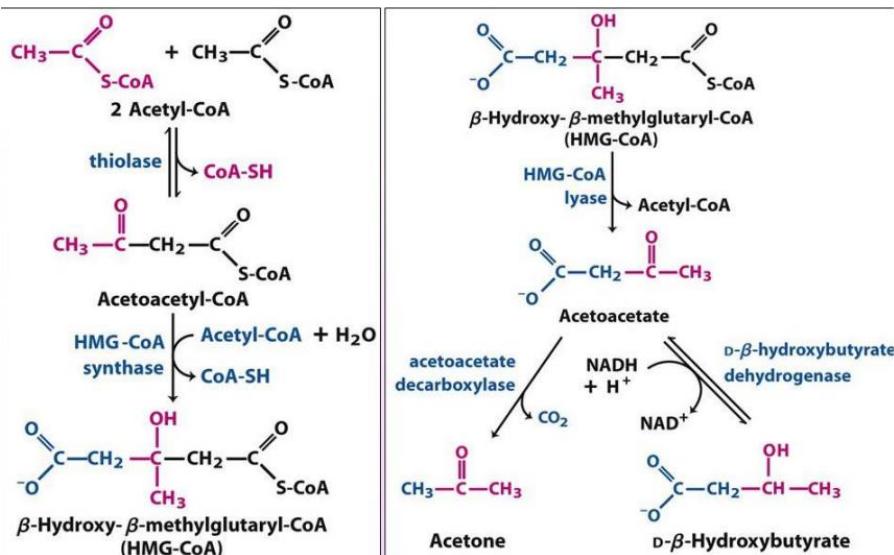
In general the ketone bodies metabolism.





Inside liver mitochondria, when we are starving, fatty acids oxidation occurs, and they form acetyl CoA. It is accumulated because oxaloacetate is limiting, and ketone bodies are synthetised. They are released in blood and transported to the other organs that need energy, in particular muscles cells of heart. Then they transform again the ketone bodies into acetyl CoA, which can enter inside the TCA cycle. In muscles, TCA is more active than liver because during starvation, gluconeogenesis is not activated so the oxaloacetate is used only for Krebs cycle, in which ketone bodies can be oxidized.

KETONE BODIES SYNTHESIS



Reactions:

- It is the inverse reaction of the last reaction of beta oxidation. 2 acetyl CoA can be condensed by thiolase to form acetoacetyl-CoA. It is reversible.
- Acetoacetyl-CoA can condensate with another molecule of acetyl-CoA to form β-hydroxy-methylglutaryl-CoA (HMG-CoA for friends – 6C). This reaction is catalyzed by HMG-CoA synthase which is the isoform we have in liver mitochondria.
- HMG-CoA is splitted to form a molecule of acetyl-CoA and a molecule of acetoacetate (first ketone bodies).
- Acetoacetate
 - Can go directly in blood (soluble in membrane and transported across it)
 - Can be decarboxylated to form acetone (volatile molecule and often it is eliminated by breath)

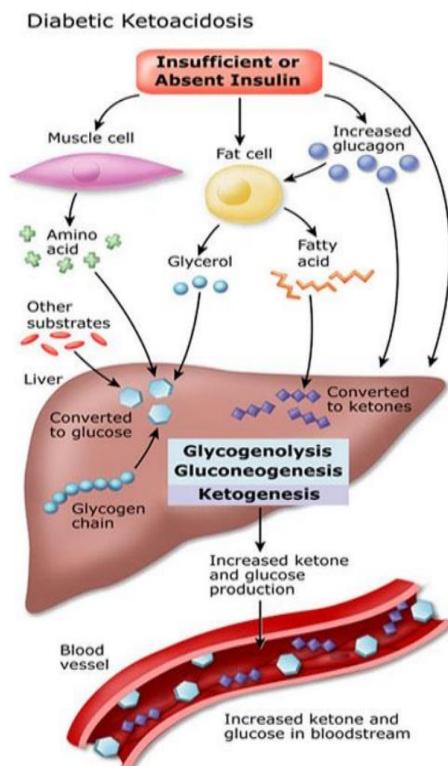
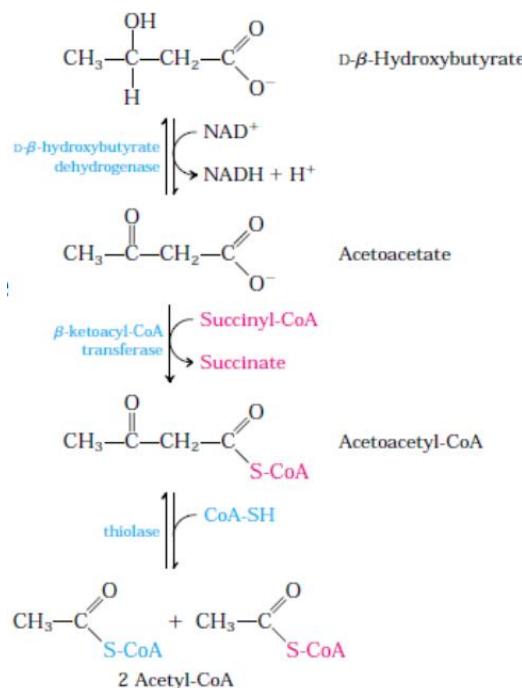
- c. Can be reduced to form beta-hydroxybutyrate (soluble in membrane and transported across it)

KETONE BODIES CATABOLISM

What happens when they are uptake by muscles (for example)? We have the reverse reactions.

1. beta-hydroxybutyrate is oxidized to form acetoacetate (we are inside to mitochondria of muscles) and NADH is directly used in respiratory chain.
2. Acetoacetate can react with succinyl-CoA to form succinate and acetoacetyl-CoA.
3. acetoacetyl-CoA is splitted by thiolase to form two molecules of acetyl-CoA, which can enter in TCA cycle.

Remember that in starving conditions ketone bodies are important for our muscles and heart to spare glucose for brain. It can use ketone bodies only when we are very starved.



In a pathologic condition like diabetes, ketone bodies can be dangerous. In fact, diabetes is not treated, in our body the concentration of glucose is not regulated. So, the concentration of glucose in blood is very high because insulin is not produced and glucose is not uptake by our cells. However, the cells in pancreas react as in starvation condition since the presence of glucose does not result. And the cells produce glucagon, which act on the liver to activate gluconeogenesis and activates the degradation of lipids and proteins in muscles. Fatty acids enter in liver, are degraded, and ketone bodies are synthetized and released. So, at the end we have an increase of ketone bodies and when they too much concentrated can induce a **ketoacidosis**, a condition in which our brain is suffering and can lead to diabetic coma.

13-Aminoacids catabolism

AMINOACIDS CATABOLISM

The oxidate phosphorylation is the real last part of catabolism for the generation of ATP. The catabolism of aminoacids is correlated with catabolism of protein and proteins are the important components of our cells, usually cells that try to spare amino acids, because the synthesis of amino acids require a lot of energy.

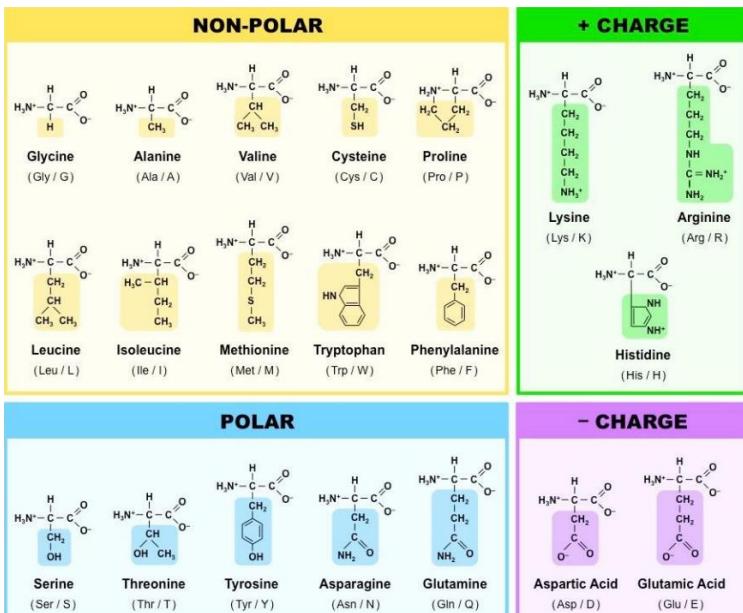
- Cells derive a small fraction of their oxidative energy from the catabolism of amino acids.

- Usually, amino acids derived from the normal breakdown of cellular proteins or from degradation of ingested proteins are directly recycled for protein synthesis. However, aminoacids contribute to the generation of metabolic energy.
- Carnivores can obtain up to 90% of their energy requirements from amino acid oxidation, whereas herbivores may fill only a small fraction of their energy. Most microorganisms can scavenge amino acids from their environment and use them as fuel. Plants rarely oxidize amino acids to provide energy.

We don't have a specific protein that is used to store for amino acids, like glycogen for glucose or lipids. So, if we have an excess of protein, in this case our cell can degraded proteins to form amino acids and can degraded amino acids to produce energy.

In animals, amino acids undergo oxidative degradation in three different metabolic circumstances:

- During the normal synthesis and degradation of cellular proteins
- When a diet is rich in protein and the ingested amino acids exceed the body's needs for protein synthesis, the surplus is catabolized because amino acids cannot be stored.
- During starvation or in uncontrolled diabetes mellitus, when carbohydrates are either unavailable or not properly utilized, cellular proteins are used as fuel.



These are all the aminoacids that we have in our proteins and their classification/structure.

Carboxylic group/amino group.

-non-polar, the chain is hydrophobic

-positive and negative

-polar, in which amino group or oxidrilic group, the chain can perform hydrogen bonds with other aminoacids or molecules.

Each aminoacids have specific metabolic pathway. One of the first step of degradation of aminoacids is the deamination.

Under all these metabolic conditions, amino acids lose their amino groups to form keto acids, the

"carbon skeletons" of amino acids. The α -keto acids undergo oxidation to CO_2 and H_2O or, often more importantly, provide three- and four-carbon units that can be converted by gluconeogenesis into glucose, the fuel for brain, skeletal muscle, and other tissues.

→The alpha keto acids can be broken/modified in different parts to form molecules that can enter in carbon hydrated pathways. They can be useful for catabolism (if cells need energy), or if cells have enough energy to activate anabolic pathway, this intermediate can be use for fatty acids synthesis or for gluconeogenesis.

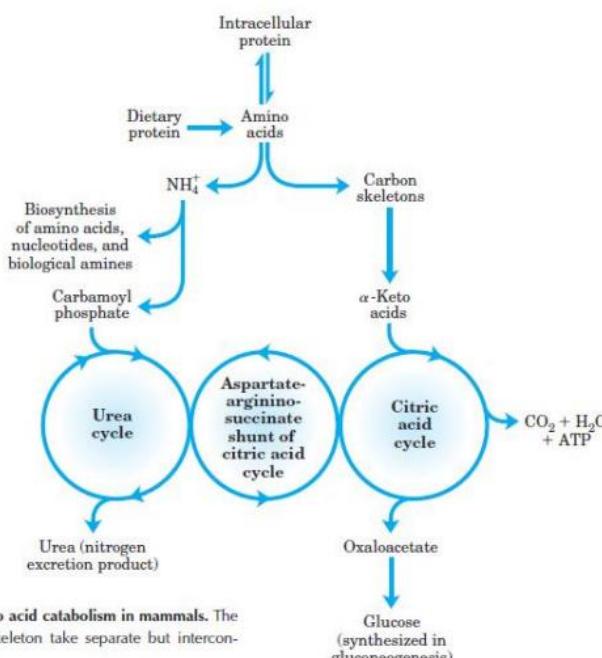
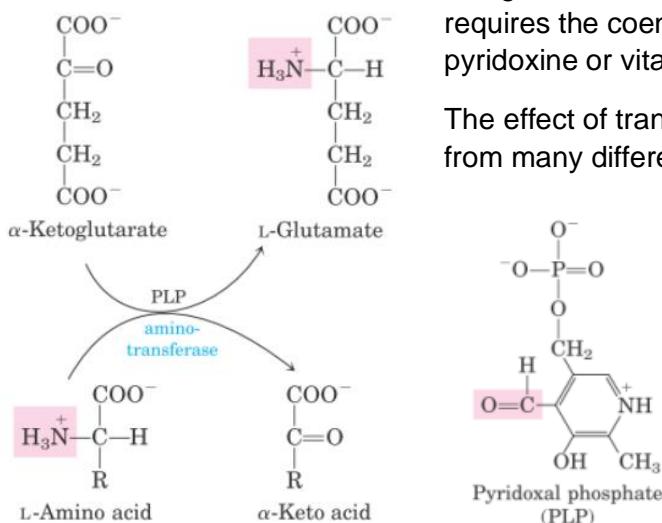


FIGURE 18-1 Overview of amino acid catabolism in mammals. The amino groups and the carbon skeleton take separate but interconnected pathways.



REACTION OF TRANSAMINATION

These enzymes have inside to the catalytic site a group that derives from vitamin B6.

This is the active part of our pyridoxal phosphate, the amino acids can react with the aldehyde group to form this intermediate.

If cells are active in their synthesis the ammonia is used again for the synthesis of other molecules that have nitrogen in their structure like nitrogen bases, for the synthesis of nucleotides or for synthesis of some sugar with nitrogen.

If the ammonia is not required because cells don't need to synthesize new molecules with nitrogen, ammonia is eliminated (because ammonia cannot be accumulated in cells, because it's toxic).

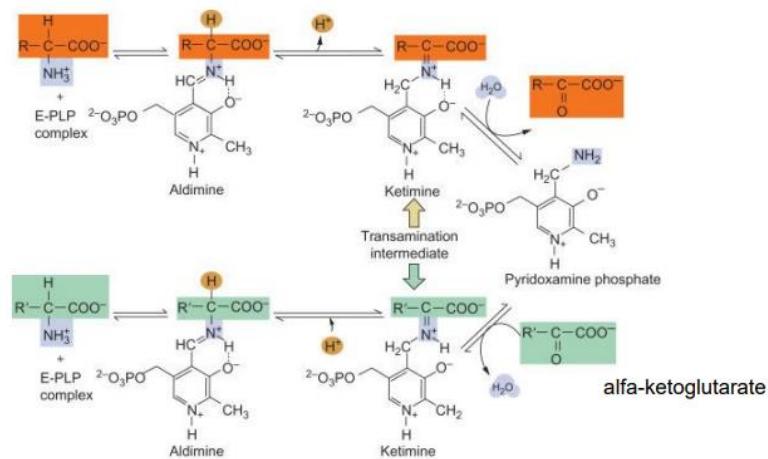
Depending on animals, but ammonia can be eliminated in different ways, in our cells is eliminated like urea.

An early step in the catabolism of amino acids is the separation of the amino group from the carbon skeleton. In most cases, the amino group is transferred to α -ketoglutarate to form glutamate. This reaction called transamination requires the coenzyme pyridoxal phosphate, the coenzyme form of pyridoxine or vitamin B6.

The effect of transamination reactions is to collect the amino groups from many different amino acids in the form of L-glutamate.

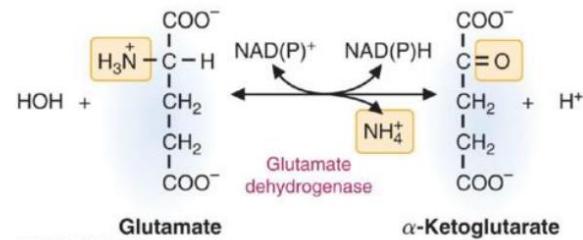
Cells contain different types of aminotransferases that differ in their specificity for the L-amino acid.

The enzymes that catalyse this reaction are called aminotransferases; they transferred an amino group. They react with 2 reactants, one that is the specific amino acids and the other is alpha ketoglutarate. (Liver is one of the organs that metabolised amino acids in our body to synthesise glucose)



Formation of glutamate

In animals, glutamate is transported to liver mitochondria, where glutamate dehydrogenase liberates the amino group as ammonium ion (NH_4^+). If for example our cells degraded proteins for producing energy, the glutamate that derives from the deamination of the amino acids is transported in blood and it's transported in liver mitochondria, in fact in liver mitochondria we have most of reactions in which our amino group is after eliminated (because it's toxic).



AMINOACIDS CATABOLISM – GLUTAMINE AS AMMONIA CARRIER

Ammonia is generated by other metabolic pathways such as nucleotides degradation in several tissues. The free ammonia produced in tissues is combined with glutamate to yield glutamine by the action of glutamine synthetase. This reaction requires ATP and occurs in two steps. After glutamine is transported in liver mitochondria and undergo to transaminat

Glutamate is one of the molecules that can transport ammonia in our body without problems (no toxic). However, another molecule is used to the transport of ammonia, Glutamine.

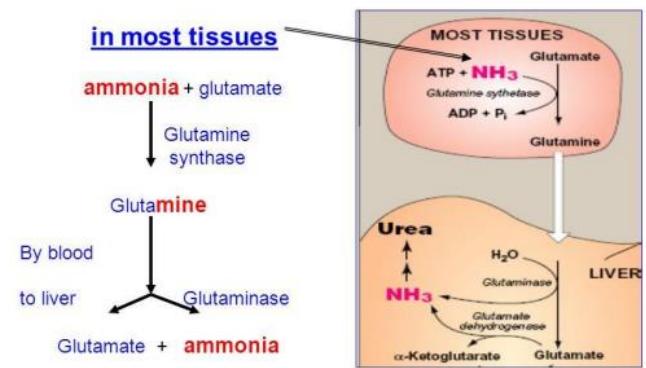
An enzyme is able to condense a second molecule of ammonia with glutamate to form glutamine, again, glutamine can be transported in blood circulation to go again in liver.

Amino acids degradation starts with the formation of glutamate, and if we have ammonia, we have also the formation of glutamine.

→ Glutamate and glutamine are the most important way to transport ammonia in our blood.

→ If cells don't need ammonia, glutamate and glutamine are oxidized to release ammonia and ammonia is eliminated to the body in different ways.

Transport of Ammonia to Liver



AMINOACIDS CATABOLISM - ALANINE AS AMMONIA CARRIER

In skeletal muscle, excess amino groups are generally transferred to pyruvate to form alanine, another important molecule in the transport of amino groups to the liver.

METABOLIC FATES OF AMMONIA

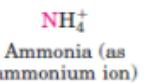
Ammonia is directly released from the tissue in the water. From terrestrial animal the problem is bigger because ammonia cannot be accumulated and it's very hydrophobic molecule and require a lot of water to be eliminated, animal have to spare water, so they have to produce molecules that are less hydrophobic and it's way they can be concentrated sparing water, this 2 molecules are: urea and uric acids.

(Urea required a lot of water to be eliminated)

Excess NH₄⁺ is excreted as ammonia (microbes, bony fishes), urea (most terrestrial vertebrates), uric acid (birds and terrestrial reptiles).

Notice that the carbon atoms of urea and uric acid are highly oxidized; the organism discards carbon only after extracting most of its available energy of oxidation.

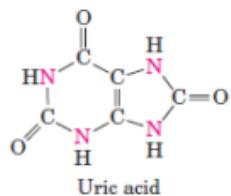
When ammonia is not still required, it is condensed with a very oxidized carbon organic molecule and it this eliminated .



Ammonotelic animals:
most aquatic vertebrates,
such as bony fishes and
the larvae of amphibia



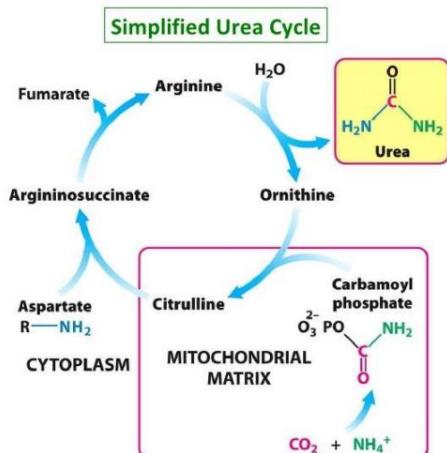
Ureotelic animals:
many terrestrial
vertebrates; also sharks



Uricotelic animals:
birds, reptiles

(b)

UREA CYCLE



The urea cycle is important because is the metabolic pathway that is present in our body.

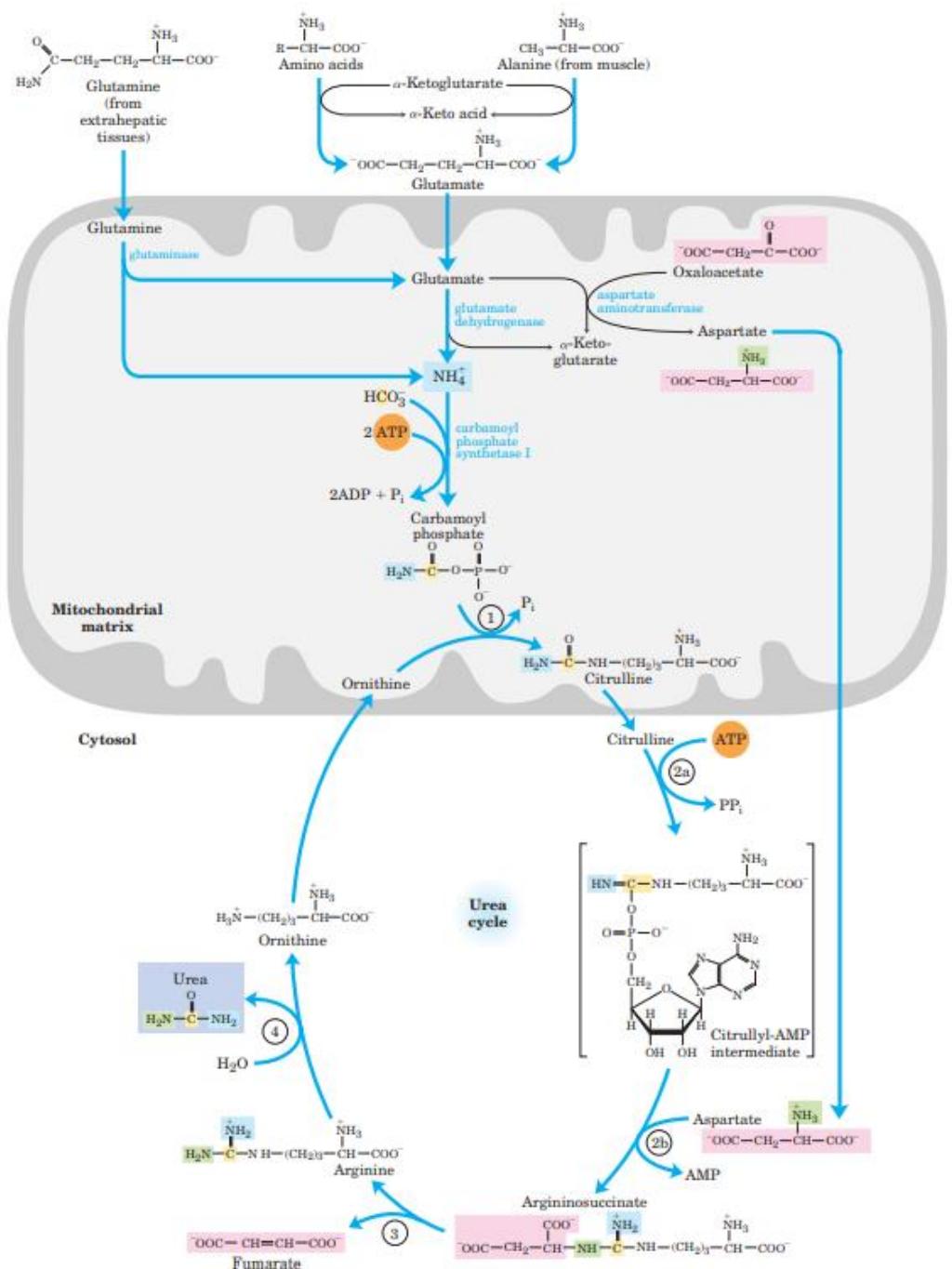
Some organisms convert ammonia to urea: the urea cycle begins inside liver mitochondria, but three of the subsequent steps take place in the cytosol. The first amino group to enter the urea cycle is derived from ammonia in the mitochondrial matrix (NH₄⁺). Whatever its source, the NH₄⁺ generated in liver mitochondria is immediately used, together with CO₂ to form carbamoyl phosphate. The carbamoyl phosphate, which functions as an activated carbamoyl group donor, now enters the urea cycle.

The cycle has four enzymatic steps. First, carbamoyl phosphate donates its carbamoyl group to ornithine to form citrulline, with the release of inorganic phosphate. Ornithine plays a role resembling that of oxaloacetate in the citric acid cycle, accepting material at each turn of the cycle: the reaction is catalysed by ornithine transcarbamoylase, and the citrulline passes from the mitochondrion to the cytosol.

The second amino group now enters from aspartate (generated in mitochondria by transamination and transported into the cytosol) by a condensation reaction between the amino group of aspartate and the carbonyl group of citrulline, forming arginosuccinate. This cytosolic reaction, catalysed by arginosuccinate synthetase, requires ATP and proceeds through a citrullyl-AMP intermediate.

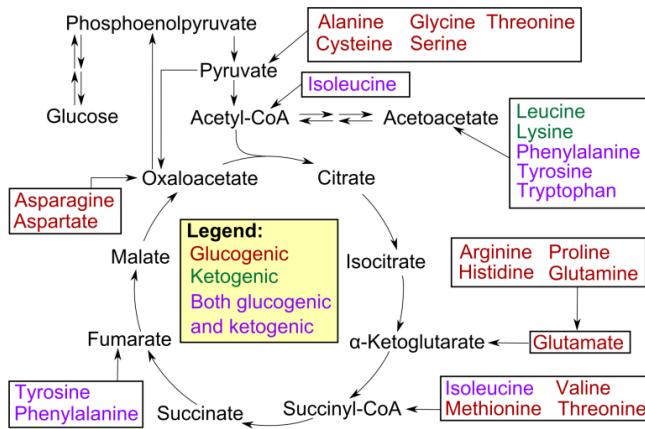
The arginosuccinate is then cleaved by arginase to form free arginine and fumarate, the latter entering mitochondria to join the pool of citric acid cycle intermediates. This is the only reversible step in the urea cycle.

In the last reaction of the urea cycle, the cytosolic enzyme arginase cleaves arginine to yield urea and ornithine. Ornithine is transported to initiate another round of the urea cycle.



The carbon skeletons of amino acids enter the citric acid cycle through five intermediates: acetyl-CoA, α -ketoglutarate, succinyl-CoA, fumarate, and oxaloacetate. Some are also degraded to pyruvate, which can be converted to either acetyl-CoA or oxaloacetate. The amino acids producing pyruvate are alanine, cysteine, glycine, serine, threonine, and tryptophan. Leucine, lysine, phenylalanine, and tryptophan yield acetyl-CoA via acetoacetyl-CoA. Isoleucine, leucine, threonine, and tryptophan also form acetyl-CoA directly.

Arginine, glutamate, glutamine, histidine, and proline produce α -ketoglutarate; isoleucine, methionine, threonine, and valine produce succinyl-CoA; four carbon atoms of phenylalanine and tyrosine give rise to fumarate; and asparagine and aspartate produce oxaloacetate. The branched-chain amino acids (isoleucine, leucine, and valine), unlike the other amino acids, are degraded only in extrahepatic tissues. A number of serious human diseases can be traced to genetic defects in the enzymes of amino acid catabolism.



OXIDATIVE PHOSPHORYLATION

It's the pathway where we have all the catabolic pathway for the synthesis of ATP, and when cells acquired ability to reduce oxygen as electron acceptor for all the catabolic pathway they can extract completely the energy from oxidation of carbon skeletal glucose but also from other metabolites and they can obtain a lot of energy.

- Oxidative phosphorylation is the culmination of energy -yielding metabolism in aerobic organisms. All oxidative steps in the degradation of carbohydrates, fats, and amino acids converge at this final stage of cellular respiration, in which the energy of oxidation drives the synthesis of ATP.
- Oxidative phosphorylation involves the reduction of O₂ to H₂O with electrons donated by NADH and FADH₂. The flow of electrons occurs through a chain of membrane-bound carriers and it is an exergonic process that is coupled to the transport of protons across a proton-impermeable membrane, conserving the free energy of fuel oxidation as a transmembrane electro-chemical potential. The transmembrane flow of protons down their concentration gradient through specific protein channels provides the free energy for synthesis of ATP.

Couple oxidate of phosphorylation, in this case we don't have the formation of an intermediate with an high energetic level, we don't have the synthesis of ATP but we have the transformation of the redox energy to the formation of proton gradient, and then the energy associated to the proton gradient is used for the synthesis of ATP.

OXIDATIVE PHOSPHORYLATION OCCURS INTO MITOCHONDRIA

- In 1948 Eugene Kennedy and Albert Lehninger demonstrated that mitochondria are the site of oxidative phosphorylation in eukaryotes.

Mitochondria derives from aerobic bacteria, that are able to perform redox reactions, oxidizing organic molecules and transporting the electron after the oxidation to oxygen and performing the oxidate phosphorylation.

- Mitochondria have two membranes. The **outer mitochondrial** membrane is readily permeable to small molecules and ions. The **inner membrane** is impermeable to most small molecules and ions, including protons, the only species that cross this membrane do so through specific transporters.
- The inner membrane bears the components of the respiratory chain and the ATP synthase.

- The mitochondrial matrix contains the pyruvate dehydrogenase complex and the enzymes of the citric acid cycle, the fatty acid oxidation pathway, and the pathways of amino acid oxidation. Among the pathways of fuel oxidation, glycolysis only takes place in the cytosol.

After the complete oxidation of a molecule of glucose by oxidative phosphorylation cells are able to synthesize 30/32 ATP, so it is useful for aerobic bacteria to produce ATP.

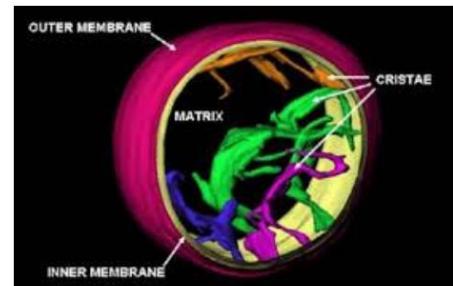
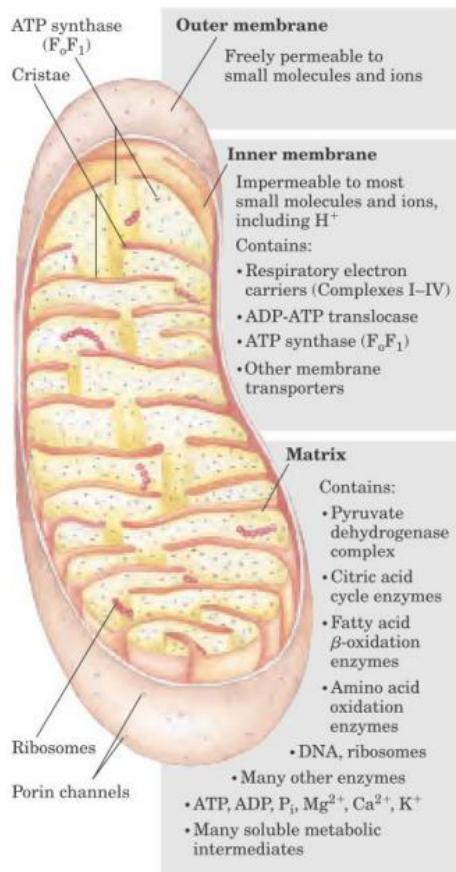
MITOCHONDRIAL STRUCTURE

- The outer mitochondrial membrane, which encloses the entire organelle has a protein/phospholipid ratio similar to that of the eukaryotic plasma membrane. It contains large numbers of integral membrane proteins called porins.

- The intermembrane space is the space between the outer membrane and the inner membrane. The concentrations of small molecules is the same as in the cytosol.

- The inner mitochondrial membrane is compartmentalized into numerous cristae, which expand the surface area of the inner mitochondrial membrane, enhancing its ability to produce ATP.

- The matrix contains a highly concentrated mixture of hundreds of enzymes, special mitochondrial ribosomes, tRNA, and several copies of the mitochondrial DNA genome. The concentration of proteins is very high.

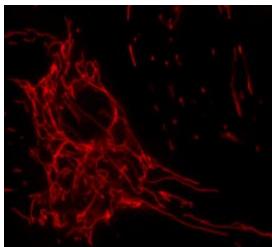


Mitochondria have their own specific code that is different from the code of the nucleus.

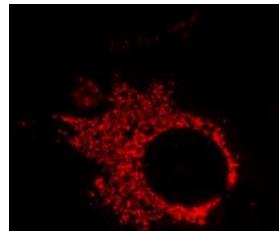
A lot of proteins are synthesized by mitochondrial DNA.

- Although commonly depicted as beanlike structures they form a highly dynamic network in the majority of cells where they constantly undergo fission and fusion, in this way they can share enzyme and product also in the matrix.
- Mitochondria vary in number and location according to cell type. Cells contain a high number of mitochondria. Human liver cells, with about 1000–2000 mitochondria per cell.
- There is a difference in concentration of protons between the inner and the matrix.

Filamentous mitochondria



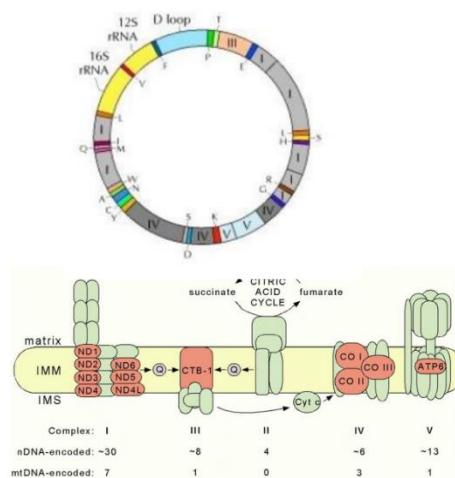
Fragmented mitochondria



In the fragmented mitochondria there is the possibility that there are some problems.

MITOCHONDRIAL GENOME

- Human mitochondrial 16 Kb genome encodes
 - Circular DNA molecule
 - Maternal inheritance
- Map
 - ✓ Origin of replication and transcriptional promoter sequences (D loop)
 - ✓ 16sRNA, 12sRNA, 22tRNA
 - ✓ 13 proteins (essential for oxidative phosphorylation)
 - ✓ Electron transfer chain complexes, including I, III, IV and V



In human in particular the genome is very compact.

Encode 2 RNA

In human, 13 proteins that are component for the respiratory chain complexes and ATP .

Subunit are the most hydrophobic sub units.

These 13 proteins are too much hydrophobic to be transported from cytosol in mitochondria, the transport is too difficult.

The proteins are synthetized inside the matrix and then inserted in the inner mitochondria membrane together with the other subunits of respiratory complexes for the synthesis of ATP.

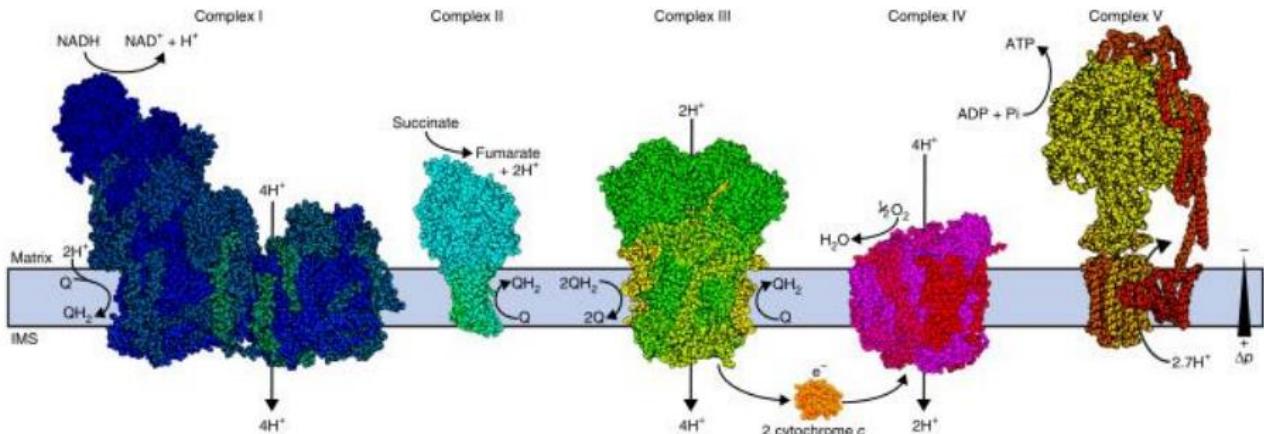
In this case mutation is very dangerous because all of these genes are extremely important for the synthesis of protein/ respiratory complexes inside the mitochondria , so the mutation is responsible for mitochondrial disease.

MITOCHONDRIAL RESPIRATORY CHAIN

The oxidation of electrons from nadh and fadh , became occurs in the inner mitochondrial membrane throw the activity of specific enzymes complex that are call complex 1,2,3,4 and 5.

The correct definition of respiratory chain is the complexes that are involved in the electron transfer.

- The electron carriers of the respiratory chain are organized into membrane-embedded supramolecular complexes.
- The respiratory complexes are connected by two mobile redox-active molecules coenzyme Q (CoQ) or ubiquinone, embedded in the membrane lipid bilayer, and a hydrophilic haeme protein, cytochrome c, located on the external surface of the inner membrane.



Complex 5 is not in the respiratory chain but in the synthesis of ATP, complex that can transform the energy of proton gradient form by respiratory chain to form ATP.

Complex oxidative phosphorylation are 1,2,3,4 and 5, but when we are talking about respiratory chain we have only 1,2,3,4, are the complex that can perform respiration, so can use oxygen.

Complex1 can oxidate all the NADH molecules that are present inside the mitochondrial matrix, and can transport electrons from NADH to a soluble carrier (another substrate) coenzyme Q.

Complex2 oxidate succinate to form fumarate, inside this enzyme we have a coenzyme molecule of fad, that is reduce to form fadh;

Coenzyme q can transport electrons to the next complex.

Only complex 3 is able to oxidate quinol and complex3 can extract electrons from quinol and transport them to the cytochrome C.

Cytochrome C can give electrons to complex 4 but also called cytochrome C oxidated .

(li spiega bene nella presentazione successiva)

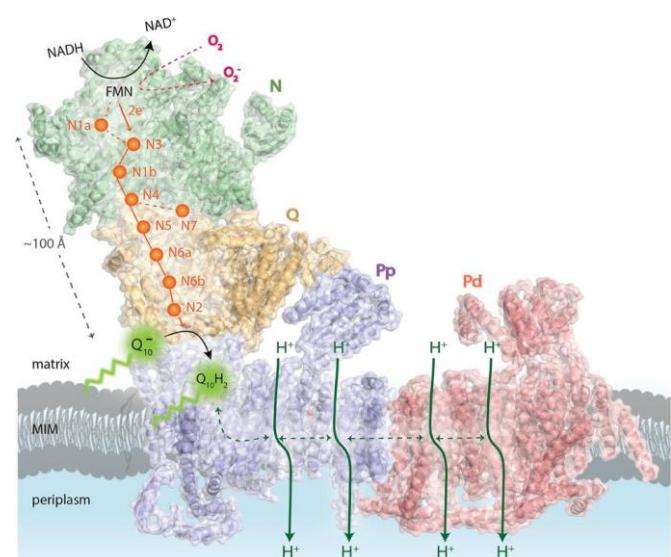
14-Oxidative phosphorylation

Mitochondrial respiratory chain: complex I

The first complex is called complex I or NADH:CoQ oxidoreductase (because this is the reaction that the complex catalyzes: oxidation of NADH and reduction of coenzyme Q). This is the most complicated enzyme in respiratory chain: in mammals it is composed by 45 subunits, whereas in bacteria by 14 subunits (also called core subunits). The minimal complex I that can work is made of 14 proteins: 7 are inside the inner mitochondrial membrane, the other 7 are present in the hydrophilic part of complex I and they content the prosthetic group of complex I.

The coenzymes in complex I are:

-Flavin mononucleotide (FMN) : it is the first acceptor of electrons from NADH, then it can donate electrons to a chain of iron sulfur centers.



-FeS centers: they form a chain of redox molecules that can transport electrons through NADH to coenzyme Q.

Coenzyme Q is present in a specific substrate of complex I.

The energy released during all these redox reactions

The energy released during all these redox reactions is used for inducing conformational changes in the subunits that are inside the membrane > this allows to open the proton channel, through which complex I can transport protons from matrix to the inner mitochondrial space.

In particular, the stoichiometry of our enzyme is: $4\text{H}^+/\text{2e}^-$: it can transport 4 protons for each couple of electrons transported from a NADH.

Mitochondrial respiratory chain: complex II

It is one of the enzymes of the Krebs cycle, it catalyzes the reaction of oxidation of succinate to form fumarate. It does this by extracting 2 electrons from succinate which are then transferred to coenzyme Q, which is again reduced ubiquinol.

Complex II is also called Succinate: CoQ oxidoreductase.

It is a simpler enzyme: it is formed by 4 subunits, all encoded in nuclear genome.

In complex II we have several coenzymes:

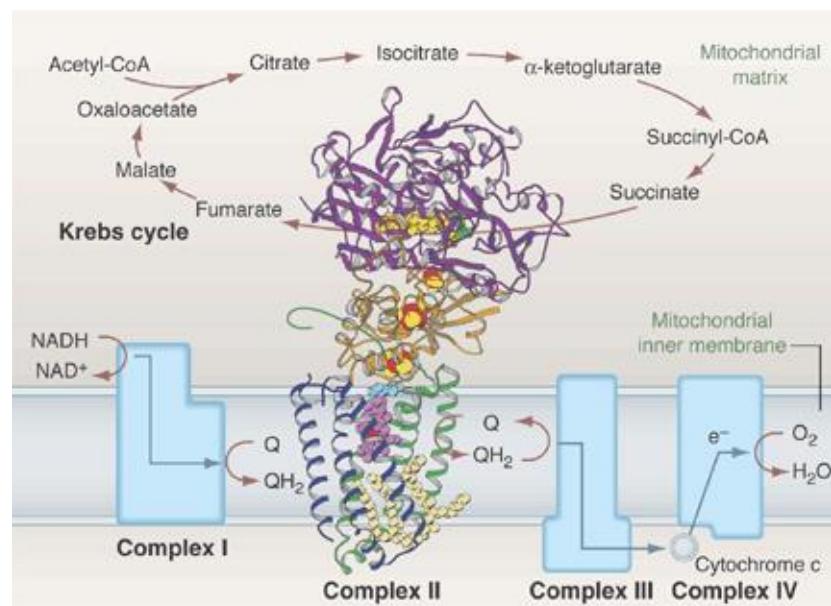
-FAD+, which is reduced to FADH₂, that is then immediately oxidized by other cofactors:

-FeS centers

-2 b-haemes > they have an organic ring (porphyrin ring), inside of which there is an ion that can be alternatively reduced and oxidized > so b-haemes are able to transport one electron per time, and they can be used in electron-transport chain.

So electrons flow from FADH₂ to iron sulfur centers b-haeme and they are transferred to coenzyme Q, which is reduced to form ubiquinol.

In this case, the energy associated to this redox reaction is not sufficient for allowing the proton pumping, so this is the only complex of the respiratory chain that is not a proton pumper, and so that cannot produce electrical proton gradient.



Mitochondrial respiratory chain: Coenzyme Q

Coenzyme Q is an electron carrier that can diffuse inside the inner mitochondrial membrane.

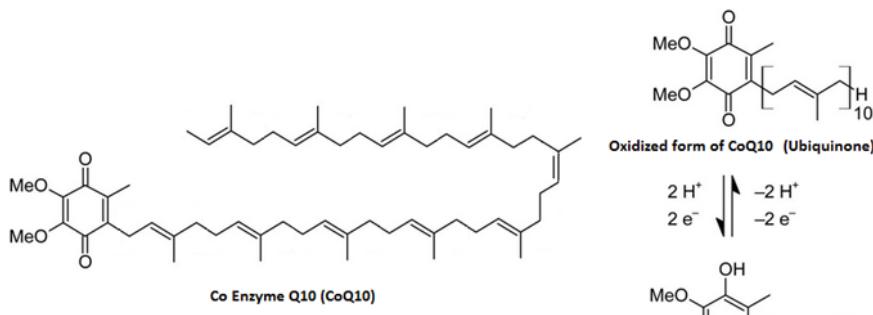
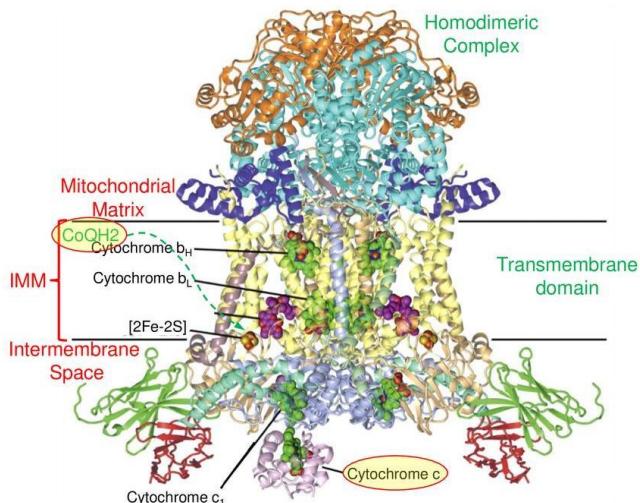
It is a very hydrophobic molecule, which has a chain formed by 10 units of isoprene (molecule with 5 carbons and double bond), so it is usually called CoQ10.

The active (redox) part of Coenzyme Q is the head, in which we have an aromatic ring with 2 carboxylic groups, that can be reduced to form hydroxylic groups.

So, Coenzyme Q can acquire 2 electrons and 2 protons to form its reduced form, also called Ubiquinol.

In cells it also has an antioxidant characteristic, because it can react with oxygen reactive species and reduce them.

Mitochondrial respiratory chain: complex III



Complex III is an enzyme that can oxidize Coenzyme Q to transfer the electrons to another acceptor, which is Cytochrome C.

It is also called CoQH₂: cytochrome c oxidoreductase.

It is a very important enzyme because all the coenzymes Q that are reduced are converged versus complex III, that is the only enzyme that can oxidize coQ. It works as an homodimer: in mammals it is formed by 20 subunits.

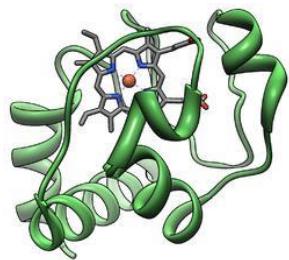
The coenzymes are: an iron sulfur center (FeS), b and c-haemes groups.

Complex III can solve the problem of transporting 2 electrons from coenzyme Q to cytochrome c, that can transport only one electron at a time. This is a problem because when coenzyme Q loses only one electron, it forms a radical, that can produce oxygen reactive species (toxic). Coenzyme Q is oxidized step by step: at the beginning it can lose only one electron (acquired by cytochrome c), then the second electron is stabilized in haeme groups to form a radical intermediate that is stabilized inside the protein. In this way, a second molecule of coQ can be oxidized, a second electron can be transported to a second molecule of cytochrome c and the other electron of coQ is used to reduce completely the radical intermediate.

This mechanism of reaction is called coenzyme Q cycle, because during all these reactions a couple of molecules of coQ are involved in a cycle of electrons. The final aim of the cycle is: to transport one electron step- by-step to cytochrome c, avoiding the formation of unstable radicals.

The stoichiometry of this enzyme is: $4\text{H}^+/\text{2e}^- = 4$ protons transported for each couple of electrons.

Mitochondrial respiratory chain: cytochrome c



Cytochrome c is an hydrophilic mobile electron carrier

It is a little molecule, in which we have a protein part and a coenzyme part : the c-haeme prosthetic group, in which we have an iron that can acquire or give an electron during redox reactions.

Cytochrome c is localized in the intermembrane space and associated to inner mitochondrial membrane.

It transports electrons from complex III to complex IV

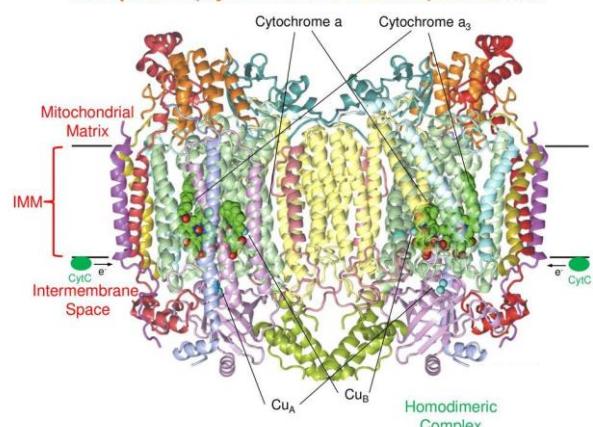
Mitochondrial respiratory chain: complex IV

Complex IV is also called Cytochrome c oxidase.

It is the real respiratory enzyme: because it is the enzyme that can reduce oxygen to form water. It is, again, active as homodimer (formed by 26 proteins in mammals).

Coenzymes are: 2 copper centers (Cu centers), that are coordinated inside the protein and can acquire and give electrons, and 2 a-haeme groups, which have inside an ion. These coenzymes are the active part of the redox reaction, and here the oxygen can enter in the enzyme and react with the electrons (given by cytochrome c) to be reduced. To completely reduce a molecule of oxygen (forming 2 molecules of water), 4 electrons are required. (so 4 cytochrome c are required). During this reduction, the complex can use the energy deriving from the redox reaction for transporting protons. In this case the stoichiometry of proton transport is: $2\text{H}^+/\text{2E}^-$: 2 protons transported across the membrane for each couple of electrons. So, at the end of all oxidations of, for example, a molecule of NADH, the 2 electrons of NADH pass through complex I, III, IV to reduce oxygen and, in these redox reactions, complex I transports 4 protons, complex III transports 4 protons and complex IV 2 protons > at the end we have the transport of 10 protons across the membrane for each couple of electrons. In the case of complex II, we skip the redox reaction of complex I > so we will have 6 protons instead of 10.

Complex IV (Cytochrome c Oxidase): Structure



Respiratory chain electron flow

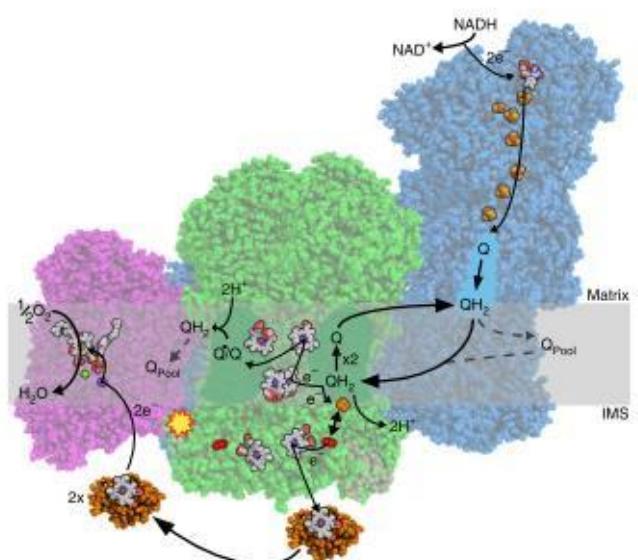
This summarizes the activity of the 3 proton pumps in respiratory electron chain: complex I, III and IV.

And we see that, from the oxidation of a molecule of NADH, we have the transport of electrons across the iron sulfur protein, then coenzyme q is reduced.

Reduced coenzyme Q is oxidized in complex III to reduce cytochrome c.

Cytochrome c can diffuse in complex IV, and complex IV can reduce oxygen to form water.

With all these reactions we have the transport of 10 protons across the membrane.



This graph is for giving us an idea of the energy involved in the redox reactions in respiratory chain.

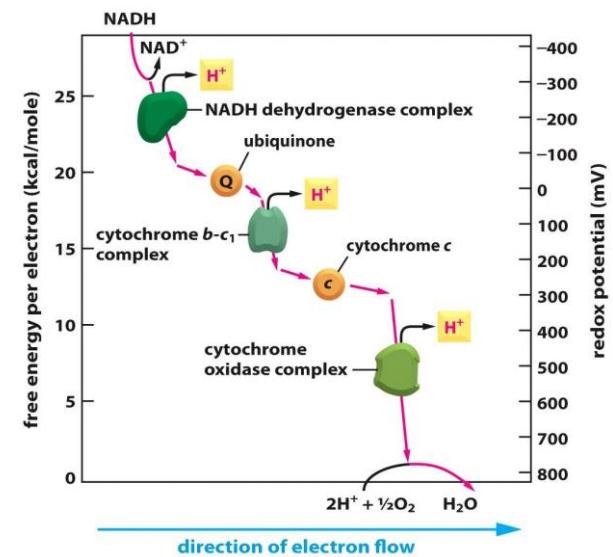
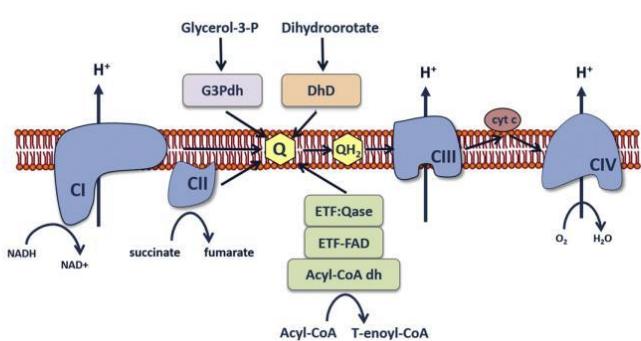
In fact, as we already know, you have a redox reaction when you have a molecule a redox potential more negative than another molecule: then the first molecule can give electrons to the molecule with higher redox potential.

We see that the differences of the redox potentials from the NADH to oxygen is very high, because NADH has potential around -400 mV, and oxygen around 800 mV.

So the difference in redox potential between NADH and oxygen is very high, and it is associated to free energy (because redox reactions are exergonic).

In fact, The free-energy change for the reaction of NADH oxidation to O₂ is 220 kJ/mol, while oxidation of succinate has a standard free- energy change of about 150 kJ/mol.

CoQ is a key node in the mitochondrial respiratory chain



Coenzyme Q is a crossroad of a lot of redox reactions, and so several enzymes, not only complex I and II, are able to reduce it.

We have already seen some of these enzymes, for example in fatty acids oxidation (ETF:Qase can transport the electrons to reduce coenzyme Q).

We also have the enzyme Glycerol-3-phosphate, which is then oxidized to glycerol dehydrogenase, which is inside the inner mitochondrial space and can, again, transport electrons to CoQ, contributing to the synthesis of ATP.

Another enzyme that we will see later is dihydroorotate dehydrogenase, involved in the synthesis of pyrimidine.

Obviously, these are accessory pathways, as the important part of the energy of cells does not derive from these pathways.

Mitochondrial respiratory chain

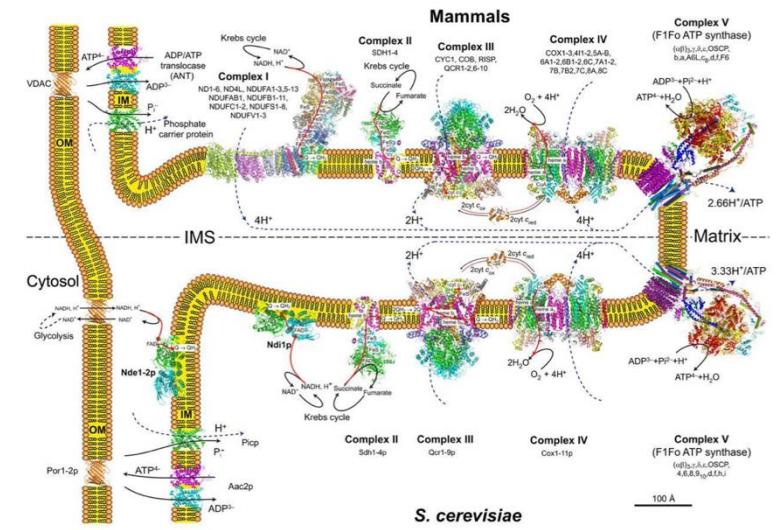
Now we will explain how the synthesis of ATP works, so how the proton gradient (formed by the respiratory complexes) is used for the synthesis of ATP.

The first thing important to know is the distribution of respiratory complexes in the mitochondrial membrane: in fact, the inner mitochondrial membrane is folded several times to form cristae, inside of which we can have the formation of proton gradient, so we will have less protons in the matrix and more in the inner mitochondrial space, in particular inside the cristae.

We have to consider that the pH in the matrix is around 8, and inside the IMS is around 6.5, so we have very different gradients of concentration of protons but also different gradient of potential, because we have accumulation of positive charges in the IMS.

When the respiratory complexes are active, protons are transported across the membrane and an electrochemical proton gradient forms across the membrane, and this is a potential energy that can be used by complex V or ATP synthase.

The respiratory complexes are usually organized in the flat of the cristae, whereas ATP synthases are usually organized in the curved part of the cristae.



Chemiosmotic theory

The hypothesis that transmembrane differences in proton concentration are the reservoir for the energy extracted from biological oxidation reactions was introduced by Peter Mitchell in 1961.

This chemiosmotic theory has been accepted as one of the great unifying principles of twentieth century biology. It provides insight into the processes of oxidative phosphorylation and into such apparently disparate energy transductions such as photophosphorylation, active transport across membranes and the motion of bacterial flagella.

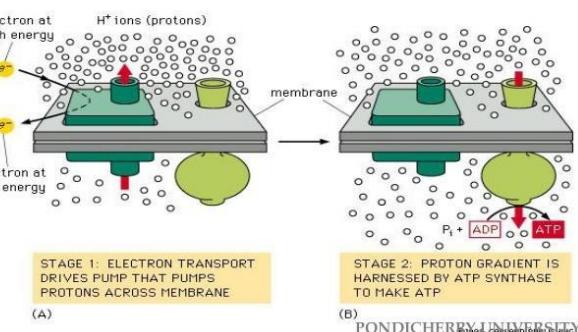
Before this theory, they already knew that ATP was synthesized in mitochondria, but they could not find an high energetic molecule that could do it.

This theory can also explains other phenomena in cells, like the synthesis of ATP in photosynthesis or the use of proton gradient for the movement of flagella.

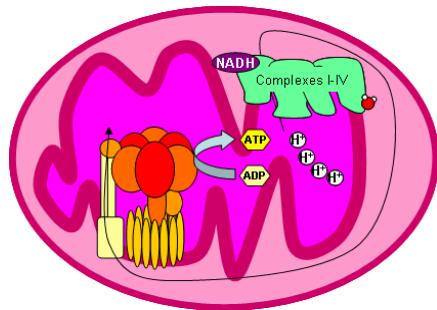
The concept of the theory is very simple: we need an impermeable membrane (like inner mitochondrial membrane) and also a system that can use chemical energy, deriving from redox reactions, to transport protons across the membrane. So, this system can couple the chemical energy, deriving from the redox reactions, to form a proton gradient and after it is important to have a protein that can use this energy for transforming it in ATP.

Chemiosmotic Coupling

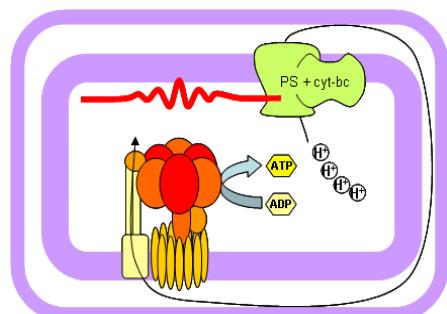
Once called the chemiosmotic hypothesis. chemi from making ATP, osmotic because of crossing the membrane. Now known as **chemiosmotic coupling**



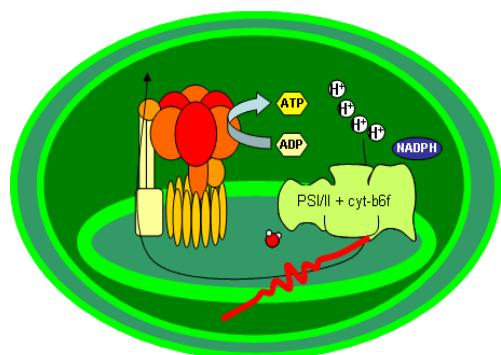
Chemiosmosis is a universal process to make ATP



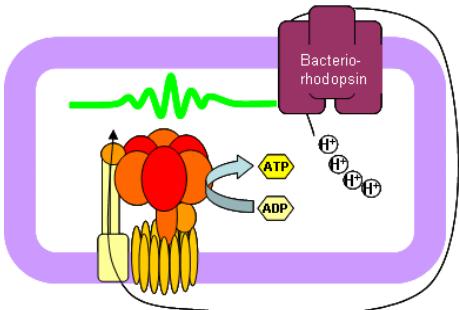
>In this case chemiosmosis is active for the synthesis of ATP in mitochondria. The respiratory complexes oxidize a NADH to produce the proton gradient, which is then used for the synthesis of ATP



>For bacteria we have photosynthetic complexes that use the light energy for starting electron transport across several donors and acceptors. Essentially the concept is the same: the energy deriving from electrons, that are excited by light and then transported across several redox transporters, is used for proton pumping and for the synthesis of ATP in bacteria.



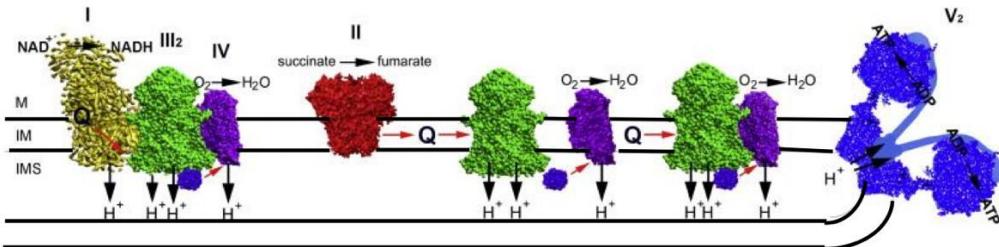
>The same process, but more complicated, occurs inside chloroplasts in plants, in which we have a photosynthetic system that is more complex. Also in this case we don't have the oxidation of NADH or FADH, but we have the energy of the light that can activate electrons, that are transported through the photosynthetic complexes and the energy deriving is used for producing proton gradient and ATP.



>In other bacteria we have a specific protein, that is not a photosynthetic protein, but is sensitive to light. It is called Bacteriorhodopsin and it is the protein from which often the pigments that give us the possibility to see derive. These proteins are sensitive to light, and in particular when it is excited by light it can transport protons and, again, allow bacteria to synthesize ATP.

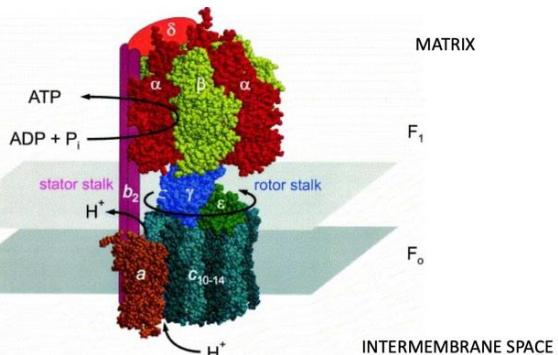
Also, in bacteria, the proton gradient is used for the movement of flagella.

The chemiosmotic theory



The free-energy change for the reaction of NADH oxidation to O₂ is 220 kJ/mol, while oxidation of succinate (or FADH in general) has a standard free-energy change of about 150 kJ/mol. Because the transfer of two electrons from NADH to O₂ is accompanied by the outward pumping of 10 H⁺, it has been estimated that roughly 200 kJ of the 220 kJ released by oxidation of a mole of NADH are conserved in the proton gradient. (from the oxidation of FADH we have the transport of 6 protons)

ATP synthase



ATP synthase is an enzyme present inside the mitochondrial membrane, and it has a part that is embedded in the membrane, which is formed by several copies of a protein called subunit C. Depending on the organism, the number of copies can vary (9 to 14/15). And these proteins form an hydrophobic ring inside the membrane. This ring is able to rotate when the protons are transferred from the inner mitochondrial space (when the concentration of protons is higher than in the matrix) and through this ring the protons can enter inside the matrix.

And the rotation of the ring is essential because it induces the synthesis of ATP.

So the ATP synthase is basically composed of 2 parts:

- the F_o part (o indicates oligomycin because it is an inhibitor of ATP synthase): formed by the C ring and in particular by the subunit gamma, which is a protein on the top of the C ring orientated versus the matrix, and which enters inside the F₁ part.
- the F₁ part: formed by several subunits: 3 alpha and 3 beta subunits, which are organized to form an hexamer and at the level of the beta subunits we have the catalytic site for the synthesis of ATP.

This part of the enzymes is connected to another part of the enzyme, called stator stalk because it is a static part (on the contrary the C ring and the gamma proteins are called rotor stalk).

Structure of Fo ATPase

We can see that we have the C ring and the gamma subunit, that is inserted inside the hexamer, and the hexamer is fixed to the membrane, because it interacts with other proteins and in particular with protein A.

The C ring is able to rotate, exploiting the proton gradient, because the structure of subunit A is specific to form 2 half-channels, one of which is open versus the inner mitochondrial membrane, so protons can enter inside it. And when protons enter inside the semi-channel, they can interact with a specific amino acid in the subunit of C ring that is an aspartic acid, which can therefore be protonated.

When the aspartic acid is protonated, in this case, it is more hydrophobic than its deprotonated form, so the subunit that faces the subunit A when it is protonated is more hydrophobic and can move out of this structure versus the membrane.

This movement allows another subunit of the C ring to face subunit A and, in this case, the subunit faces the semi-channel in the matrix.

In this semi-channel, our protonated aspartic acid can be deprotonated, because we are in a hydrophilic environment in which the concentration of protons is low and so the dissociation of the carboxylic group is favored, and the protons can exit from the semi-channel.

In this way the protons can enter inside the membrane from one hemichannel and can exit versus the matrix thanks to other hemichannel, and this movement can rotate the C ring.

The demonstration that the C ring was able to rotate inside the membrane was done through this experiment:

They purified ATP synthase, they blocked part of it to a substrate, and they attached to subunit C a fluorescent filament of actin to see the rotation.

ATP synthase is able to perform the reaction of synthesis of ATP using the proton gradient, but it is also able to use the energy from the hydrolysis of ATP to create the proton gradient in a reverse way.

So, the researchers gave ATP to ATP synthase, which was able to hydrolyze it into ADP and the energy of the hydrolysis was used to perform the opposite reaction, and the result was the rotation of the C ring.

Structure of F1 ATPase

The rotation of the C ring also induces the rotation of the gamma subunit, which also induces several conformational changes that are important for the synthesis of ATP.

In particular the beta subunits are the ones in which we have the catalytic sites for the synthesis of ATP. (catalytic subunits)

The alpha subunits are homologs of beta subunits but are modified because they cannot synthesize ATP but they interact with nucleotides or other molecules to regulate the activity of ATP synthase. (regulatory subunits)

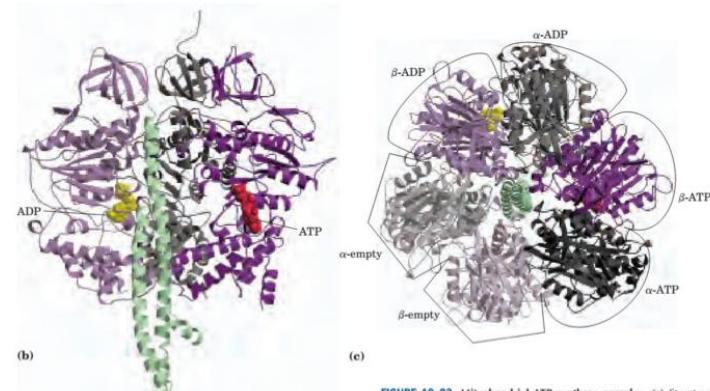
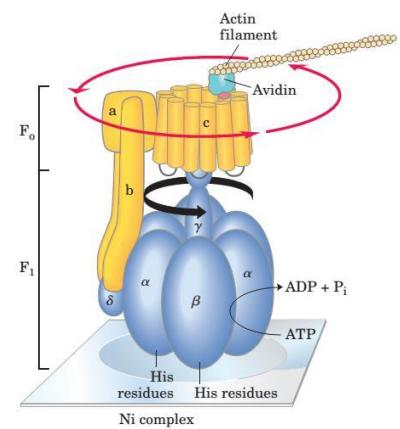
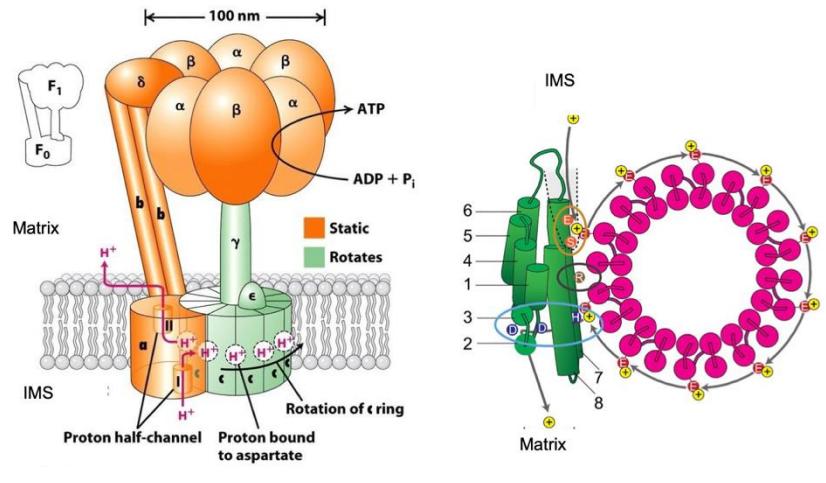
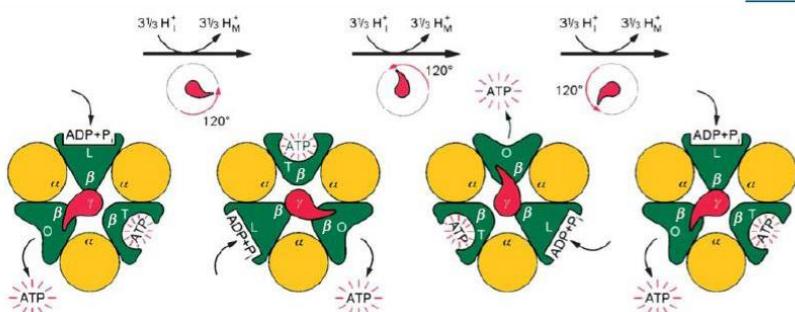


FIGURE 19-23 Mitochondrial ATP synthase complex. (a) Structure

Binding-change model for ATP synthase

The mechanism for the synthesis of ATP was explained by this model, in which it is considered

that the three beta subunits exist in three distinct conformations:



synthesis of ATP, catalytic site closed)

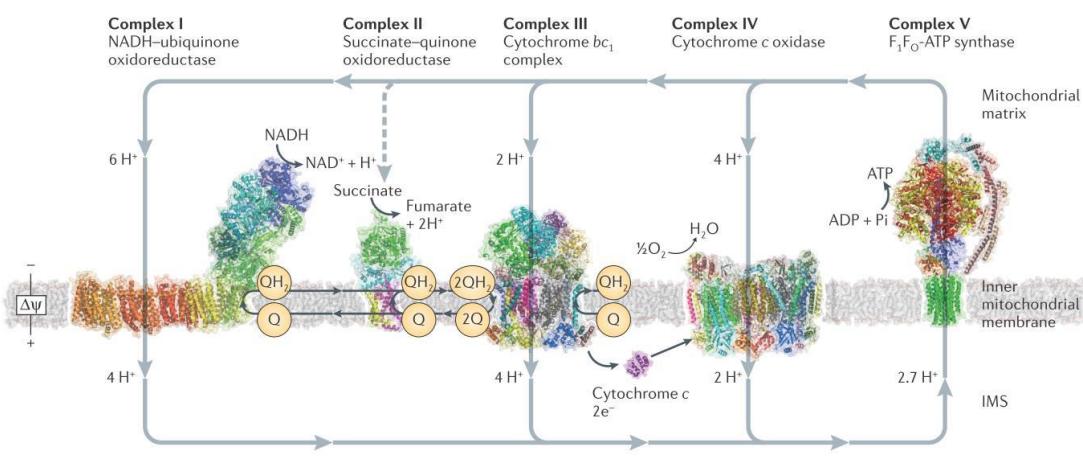
□ O (open structure) in which ATP has low affinity and it is released (the catalytic site is completely open)

The alternative modification of these structures for each subunit is the mechanism in which ATP synthase can synthesize ATP. (loose > tight > open)

The change of these 3 structures for each beta subunit is induced by the movement of the gamma subunit (rossa nel disegno)

It has been estimated that for inducing a rotation of 120 degrees (so from a beta subunit to another) it is required the transport of 3 protons. > this means that to synthesize a molecule of ATP we need the passage of 3 protons.

Stoichiometry of oxidative phosphorylation



Stoichiometry of ATP synthesis is not exactly recognized

Most experimental measurements indicate that 10 H⁺ are transported across the membrane after NADH oxidation and 6H⁺ for FADH₂ oxidation

and a 1/2 O₂ is reduced. About three protons are required to synthesize an ATP.

Thus, for each NADH it has been estimated a P/O ratio of 3 and for each FADH₂ a P/O ratio of 2.

(this estimation assumes perfect condition with no errors)

In bacteria this is true, but in mitochondrial is lower: NADH produces about 2.5 ATP and 1 FADH₂ produces 1.5 ATP

15-Oxidative phosphorylation

STOICHIOMETRY OF OXIDATIVE PHOSPHORYLATION

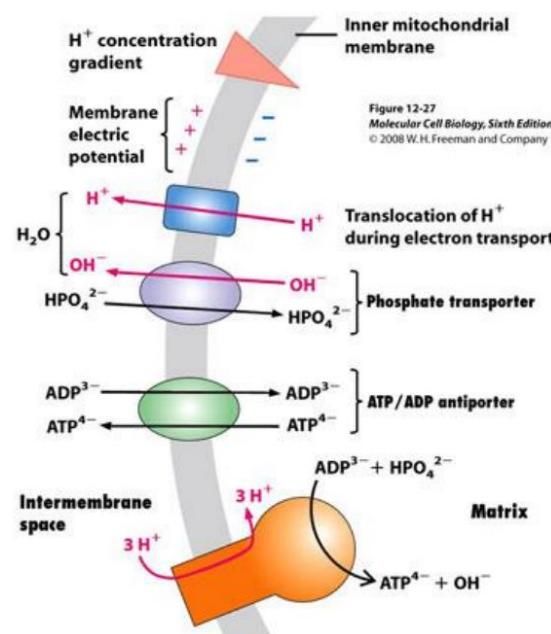
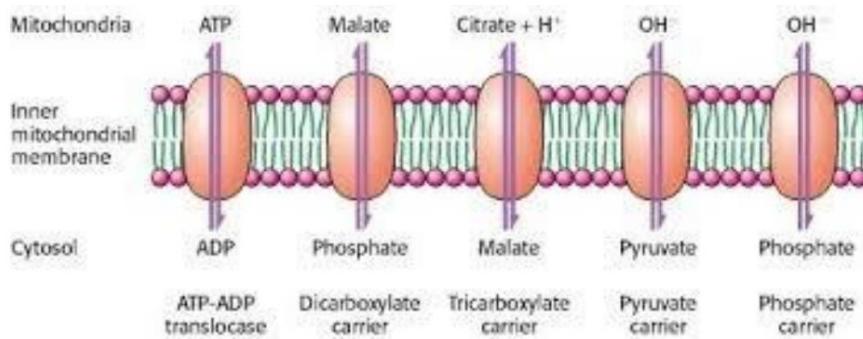
all of the proton gradient is used for the synthesis of ATP through the oxidation of a molecule of NADH, we have the synthesis of 3 ATP (10 protons are transported across the respiratory complex). From the oxidation of a molecule of FADH from complex 2 but also from the other complexes that transfer electrons to coenzyme Q. In this case we have the transport of 6 p+ (protons) and 2 ATP. This is true for bacteria in which the oxidative phosphorylation complexes are in the plasma membrane and more or less all the p+ gradient is used for the synthesis of ATP.

In mitochondria the stoichiometry is a little bit different in fact through the inner mitochondrial membrane, that is impermeable to metabolites, substrates, p+s and ions, the transport of all of these molecules is made by carriers. But these carriers often use p+ gradient as force for the transport of pyruvate and other metabolites, and also ATP and ADP, through the inner mitochondrial membrane.

The transport of ATP and ADP consumes p+ gradients because the ATP that is synthesized inside to the mitochondria is transferred in cytosol (for anabolic pathway and exergonic reactions) and at the same time with the same carrier, which is an antiporter, we have

So in this case we have the exit of ATP with 4 negative charges at cellular ph and the entrance of ADP with 3 negative charges. In this way, a part of the negative charge that is transferred outside dissipate a little bit the p+ gradient.

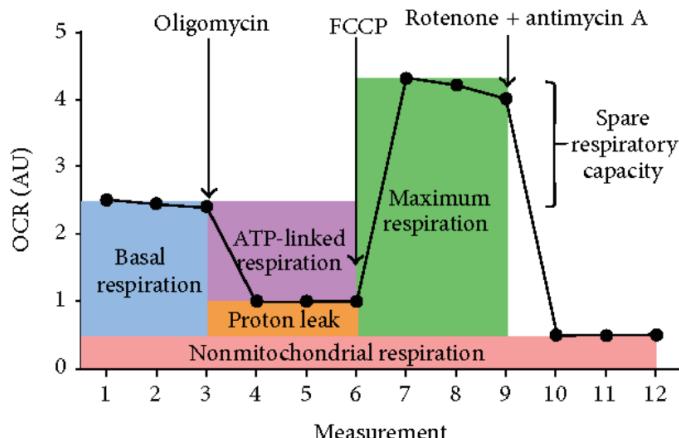
For each molecule of ATP we lose a p+ and the balance of synthesis of ATP in mitochondria is considered 2.5 ATP for each NADH molecule oxidized and 1.5 ATP for each FDH2 molecule oxidized. Not only because we have ATP and ADP antiporter, but also because we have other carriers:



(She literally reads the image)

There are many carriers that use the force associated to the p+ gradient to transport against the gradient molecules. The number of molecules used for synthesis of ATP are less than 2 or 3 (depending on FAD2 or NADH) because a part p+ gradient is used by other carriers.

THE ACTIVITY OF RESPIRATORY CHAIN IS SYNCHRONIZED WITH ATP SYNTHESIS: THE RESPIRATORY CONTROL



The oxidative regulation is regulated by phosphorylation, concentration of calcium but the most important is the strict connexion between ATP synthase and respiratory chain (they are coupled). The rate of activity of the respiratory chain, strictly depends on the ability of ATP synthase to dissipate the p^+ gradient: if ATP synthase is active, e-s are transported from the donors (FADH₂ and NADH) to oxygen and the free energy associated is enough to transporting due p^+ s across the membrane; if ATP synthase slows down, also the dissipation of p^+ gradient slows down. In this case, the p^+ gradient is accumulated in the inner mitochondrial membrane and will be too much, and the free energy will not be enough for transport the p^+ s against the gradient.

This regulation is called **respiratory control**: the respiratory chain transports e-s and catalyses redox reactions in a rate that depends on the dissipation of the p^+ gradient that depends on the activity of ATP. This is important because if cells need a lot of ATP, ADP enters inside to mitochondria, the ATP synthase dissipates membrane potential, and the respiratory chain is activated. When cells do not need ATP, ATP synthase slows down its activity as the respiratory chain slows down the consumption of oxygen. So, we can measure the respiratory control by measuring the oxygen concentration consumed by mitochondria in cells, using specific instruments.

For example:

Basal respiration: when cells are in basal conditions, they O₂ consumption is very low.

If we have the inhibitor of ATP synthase (oligomycin), the c-ring is stopped, and the ATP synthase cannot transport p^+ s for the synthesis of ATP. And so, you can see that the O₂ consumption is coupled with the ATP synthesis: **ATP-linked respiration:** when you the synthesis of ATP, the O₂ reduction is reduced. This reduction corresponds to the O₂ used by cells to synthesise ATP.

If we add an uncoupler (FCCP), a molecule that can dissipate the p^+ gradient (usually creates holes in the membrane), and in this way the membrane is permeable to p^+ s, which try to balance the concentration outside and inside. In this case the p^+ gradient is dissipated, and the respiratory chain tries to recreate it and so it goes at its maximum possibility (**maximum respiratory**): the complexes are very active because they try to oxidize NADH and FADH₂ and reduce O₂ to recreate the p^+ gradient, but it is not possible because the uncoupler continuously dissipates p^+ gradient.

Note: the basal respiration should always be less than the maximum respiration. It is important because mitochondria can increase or decrease respiration depending on the activity of ATP synthase.

We can block completely the respiration by adding inhibitors of the respiratory complexes (Rotenone + antimycin A). The only amount of O₂ that is consumed in presence of inhibitors is called **nonmitochondrial respiration**, in fact cells can use O₂ in other ways (not related to ATP).

Proton leak corresponds to the transport of p+ through membrane due to the carriers. When we have carriers that are very active, they use a significant amount of p+s for the transport of metabolites and in this case the respiratory chain tries to maintain the p+ gradient consuming O₂. This has explained us why not all the p+ gradient and the O₂ consumed is used for the synthesis of ATP.

How cells can transport e-s derived from the oxidation of glucose in glycolysis in cytosol and how can they use them in respiratory phosphorylation?

In glycolysis you have the production of NADH, which cannot be directly oxidized by oxidative phosphorylation. In aerobic organisms, NADH is oxidized in mitochondria but it cannot be directly transported inside, it needs a **carrier**. To transport e-s from the cytosol to mitochondria, cells use two shuttle: malate aspartate shuttle and the α -glycerophosphate dehydrogenase shuttle.

Malate aspartate shuttle

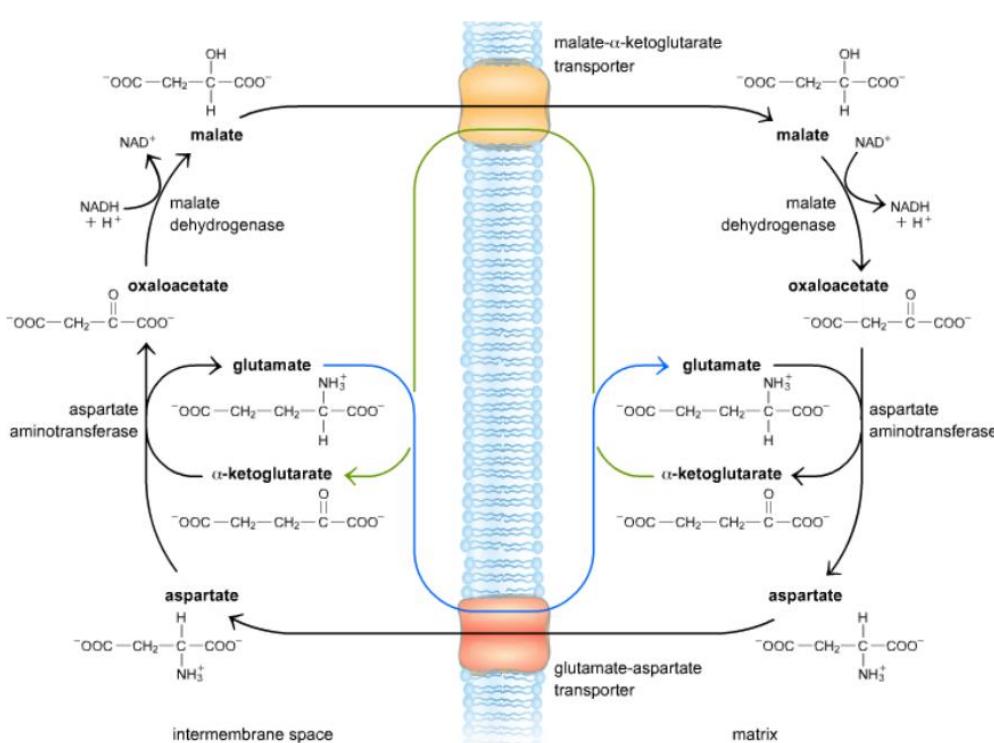
It is the most used by cells. It uses different molecules, in particular they have the carrier that transport them.

So, we have NADH in cytosol, it can be oxidized by the enzyme called malate dehydrogenase, which is an isoform (similar to the enzyme in Krebs cycle). Malate is a molecule that has a carrier, in particular an antiporter, in fact malate can be transported thanks to the malate alpha-ketoglutarate transporter. It makes an exchange with a molecule of alpha-ketoglutarate, a molecule present at high concentrations inside mitochondria. Malate now can be oxidized by the last step of Krebs cycle, to form oxaloacetate. In this way, e-s deriving from NADH are transported into mitochondria and we have the formation of a molecule of NADH, which is directly oxidized by complex I.

But we have to create a cycle. The fate of oxaloacetate is used for a lot of reactions and it is also used for the malate aspartate shuttle. Note: it is not a metabolic pathway because we don't have

the synthesis of new molecules but only a recycling. Final goal: transport e-s inside mitochondria.

So oxaloacetate can be aminated by its specific transaminase into aspartate and the donor is glutamate. Glutamate is deaminated to form alpha-ketoglutarate. Because there isn't a direct transporter for oxaloacetate in mitochondria membrane. On the contrary, there is a transporter for aspartate called glutamate-aspartate transporter, again an

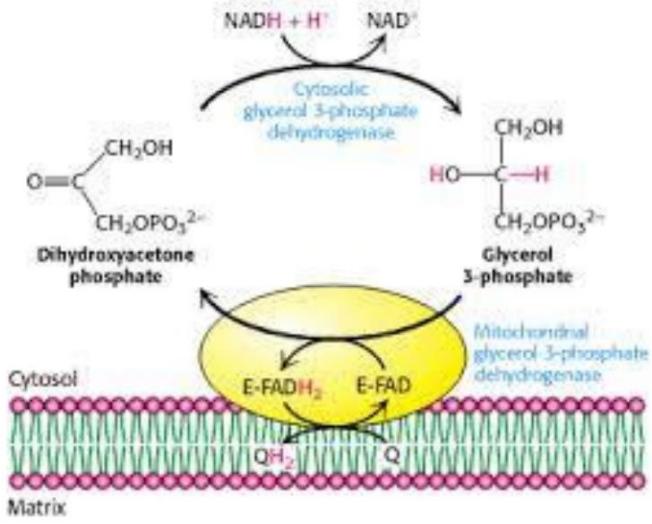


antiporter: for each molecule of aspartate transported outside, a molecule of glutamate is transported inside.

So aspartate is deaminated into oxaloacetate. The alpha-ketoglutarate, that exits through the above transporter, is aminated into glutamate.

All of these reactions are reversible but the shuttle goes in the direction described above because when glycolysis is active, the concentration of NADH in cytosol is very high and so all the reactions are shifted versus the formation of malate and the exit of aspartate. But, if for any reason the concentration of NADH is reduced in cytosol, e-s can be transported in the reverse way.

α -glycerophosphate dehydrogenase shuttle



It is allowed by two enzymes that have the same name: glycerol 3-phosphate dehydrogenase, but one is cytosolic and the other one is mitochondrial. The first one is in cytosol, and it is completely soluble while the second one is associated with the inner mitochondria membrane, similarly to complex II, but the catalytic site is in the inner mitochondrial space.

We have a dihydroxyacetone phosphate, that derives from glycolysis, and it can be released by using NADH. The reduction produces a glycerol 3P, which can enter inside in the matrix and react with the membrane protein. The oxidation

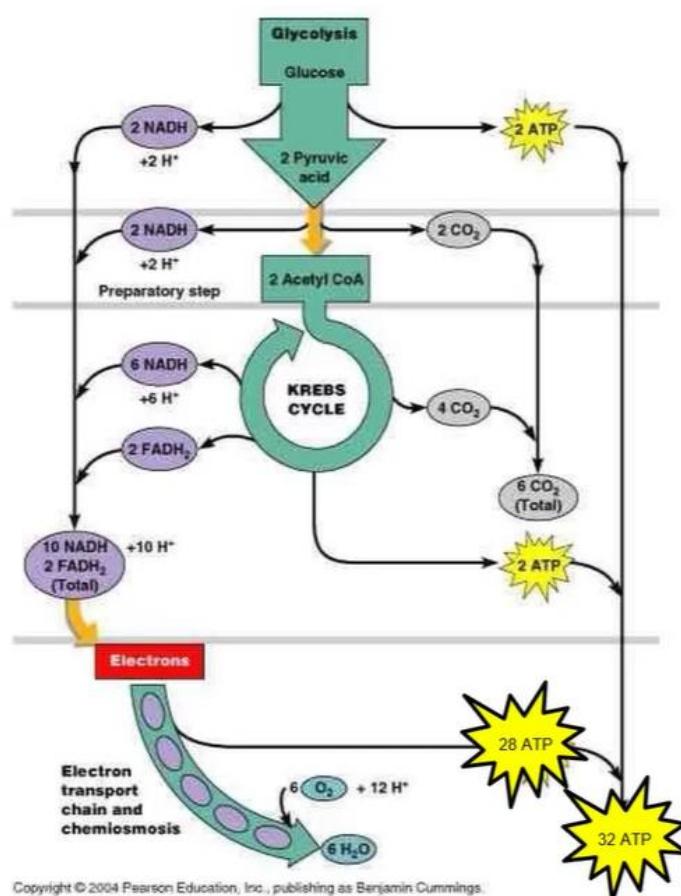
produces again the dihydroxyacetone phosphate and the e-s from NADH are transported to a coenzyme (E-FAD) inside to an enzyme (yellow circle) and it is reduced into E-FADH₂. Now, mitochondrial glycerol 3-phosphate dehydrogenase is a membrane enzyme that can transport the e-s from FADH₂ to coenzyme Q directly, which can be oxidized by complex III, IV, ... In this case, we have the transport of 6 p+s. This means that if cells use the shuttle, a part of energy present in NADH, is lost because during this redox reaction we have the formation of FADH₂ and we have the transport of only 6 p+s and not 10.

So the malate aspartate shuttle maintain the energy of NADH completely intact (2.5 ATPs) while for α -glycerophosphate dehydrogenase shuttle the production ATP corresponds to 1.5 ATPs.

The second shuttle is not often used, these enzymes are enzymes that can be used in cells depending on the metabolism of cells.

These are exergonic reactions and irreversible.

ENERGETIC BALANCE OF GLYCOLYSIS



$28\text{ATP} + 2\text{ATP}$ from Krebs cycle + 2ATP from anaerobic glycolysis = 32ATP complete oxidation of glycolysis.

This scheme gives us a summary of all of the reactions we have seen with the production of ATP. Note: we consider 32ATP if the malate aspartate shuttle is working otherwise is 30ATP.

Glycolysis is considered a very efficient mechanism for synthetizing ATP

Anaerobic glycolysis: 2ATP (glucose \rightarrow pyruvate)

Aerobic glycolysis: pyruvate is completely oxidized inside mitochondria to give 2NADH . Then acetyl CoA enters in Krebs cycle and for each cycle we have the formation of 3NADH , 1FADH_2 and 1GTP (or ATP depending on the organism). Then multiply them for 2: 6NADH , 2FADH_2 and 2GTP . Now, all these molecules, are used in the respiratory chain to reduce O_2 to H_2O and to synthetize ATP. At the end of the oxidation of glucose we have: 10NADH , 2FADH_2 .

If you consider the production of ATP associated to this molecule in very perfect conditions in which most of redox energy associated to NADH and FADH₂ is used for the synthesis of ATP, we can consider that from 10NADH we have the formation of 25ATP (10×2.5) and for 2FADH_2 we have 3ATP (2×1.5). At the end we have 28ATP from the all the reduced nucleotides.

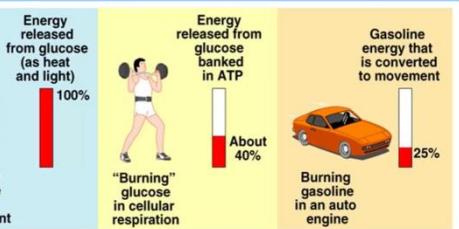
TABLE 16-1 Stoichiometry of Coenzyme Reduction and ATP Formation in the Aerobic Oxidation of Glucose via Glycolysis, the Pyruvate Dehydrogenase Complex Reaction, the Citric Acid Cycle, and Oxidative Phosphorylation

	Reaction	Number of ATP or reduced coenzyme directly formed	Number of ATP ultimately formed*
1			
2			
3	Glucose \longrightarrow glucose 6-phosphate	-1 ATP	-1
4	Fructose 6-phosphate \longrightarrow fructose 1,6-bisphosphate	-1 ATP	-1
5	2 Glyceraldehyde 3-phosphate \longrightarrow 2 1,3-bisphosphoglycerate	2 NADH	3 or 5†
6	2 1,3-Bisphosphoglycerate \longrightarrow 2 3-phosphoglycerate	2 ATP	2
7	2 Phosphoenolpyruvate \longrightarrow 2 pyruvate	2 ATP	2
8	2 Pyruvate \longrightarrow 2 acetyl-CoA	2 NADH	5
9	2 Isocitrate \longrightarrow 2 α -ketoglutarate	2 NADH	5
10	2 α -Ketoglutarate \longrightarrow 2 succinyl-CoA	2 NADH	5
11	2 Succinyl-CoA \longrightarrow 2 succinate	2 ATP (or 2 GTP)	2
	2 Succinate \longrightarrow 2 fumarate	2 FADH ₂	3
	2 Malate \longrightarrow 2 oxaloacetate	2 NADH	5
	Total		30-32

* This is calculated as 2.5 ATP per NADH and 1.5 ATP per FADH₂. A negative value indicates consumption.

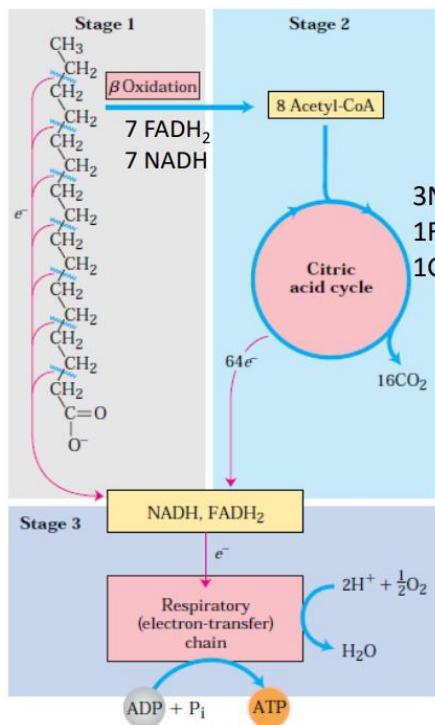
† This number is either 3 or 5, depending on the mechanism used to shuttle NADH equivalents from the cytosol to the mitochondrial matrix; see Figures 19-27 and 19-28.

because if you consider the energy deriving from the complete chemical oxidation of glucose, the free energy associated is 2849 kJ/mol. If we consider 30-32ATP, the energy is around 900-980 kJ/mol. And if we consider the efficiency of this process, about 40% of the energy present inside glucose, can be used for synthetizing ATP. 60% is lost as heat.



If you compare the efficiency of aerobic glycolysis to the energy needed to move a car, is less efficiency than our mitochondria.

ENERGETIC BALANCE OF FATTY ACIDS OXIDATION



1 mole of glucose produces 30-32 moles ATP $\rightarrow \approx 900-980$ kJ

1 mole of palmitate produces 108 moles ATP $\rightarrow \approx 3300$ kJ

Molecular Weight of glucose 180 g/mol; MW of palmitate 256 g/mol

For producing 3300 KJ with glucose, we need of about 590gr of glucose.

Ex palmitic acid (16C)

7 cycles of degradation \times (1 step formation FADH₂ and 1 step formation NADH) = 7 FADH₂ and 7 NADH

Then, the 8 acetyl-CoA can enter the citric acid cycle: (3 NADH, 1 FADH₂, 1GTP) \times 8 = 24 NADH, 8FADH₂, 8GTP

15 FADH₂ \times 1.5ATP = 22.5 ATP

31 NADH \times 2.5 ATP = 77.5 ATP

8 GTP = 8 ATP

TOT 108 ATP

This is the reason for which our best energetic store are fatty acids and not glucose or glycogen, because the energy derived from the oxidation of fatty acids, in particular saturated ones, is higher than the oxidation of glucose. It may be slower but for efficacy.

TABLE 17-1 Yield of ATP during Oxidation of One Molecule of Palmitoyl-CoA to CO_2 and H_2O

Enzyme catalyzing the oxidation step	Number of NADH or FADH ₂ formed	Number of ATP ultimately formed*
Acyl-CoA dehydrogenase	7 FADH ₂	10.5
β -Hydroxyacyl-CoA dehydrogenase	7 NADH	17.5
Isocitrate dehydrogenase	8 NADH	20
α -Ketoglutarate dehydrogenase	8 NADH	20
Succinyl-CoA synthetase		8 [†]
Succinate dehydrogenase	8 FADH ₂	12
Malate dehydrogenase	8 NADH	20
Total		108

*These calculations assume that mitochondrial oxidative phosphorylation produces 1.5 ATP per FADH₂ oxidized and 2.5 ATP per NADH oxidized.

[†]GTP produced directly in this step yields ATP in the reaction catalyzed by nucleoside diphosphate kinase (p. XXX).

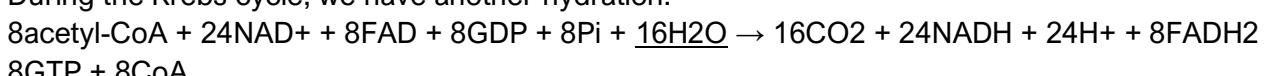
METABOLIC WATER FROM FATTY ACIDS OXIDATION

The oxidation of fatty acid is also an important source of metabolic H_2O . In fact, at the end of the oxidation of NADH and FADH₂, we have the reduction of O_2 to produce H_2O . And the water that it is produced during the oxidative phosphorylation is called **metabolic water**, which can be used in cells for other metabolic pathways. Ex above, Palmitoyl-CoA

At the beginning, H_2O is consumed because we have an hydration:



During the Krebs cycle, we have another hydration:



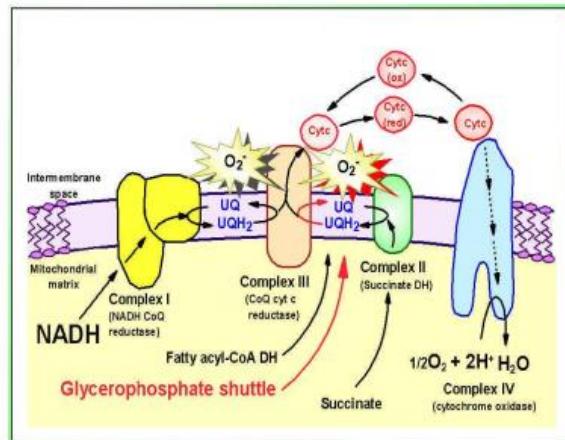
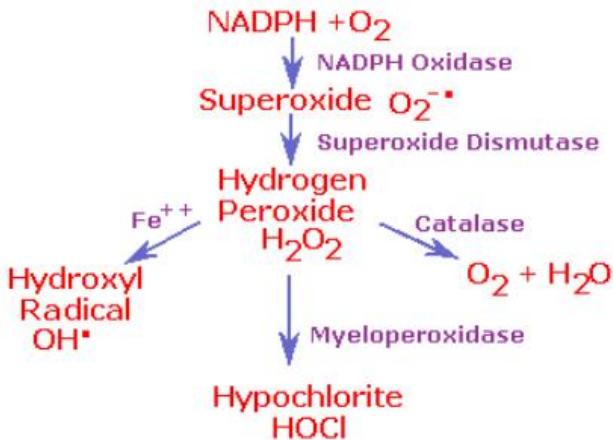
But during oxidative phosphorylation, the respiratory chain can reduce several molecules of O_2 to produce H_2O :



$$\text{TOT: } 46\text{H}_2\text{O} - (7+16)\text{H}_2\text{O} = 23\text{H}_2\text{O}$$

This water is very important for animals such as camels. They use the fatty acids stored in humps to give energy and water.

RESPIRATORY CHAIN IS THE MAJOR SOURCE OF REACTIVE OXYGEN SPECIES

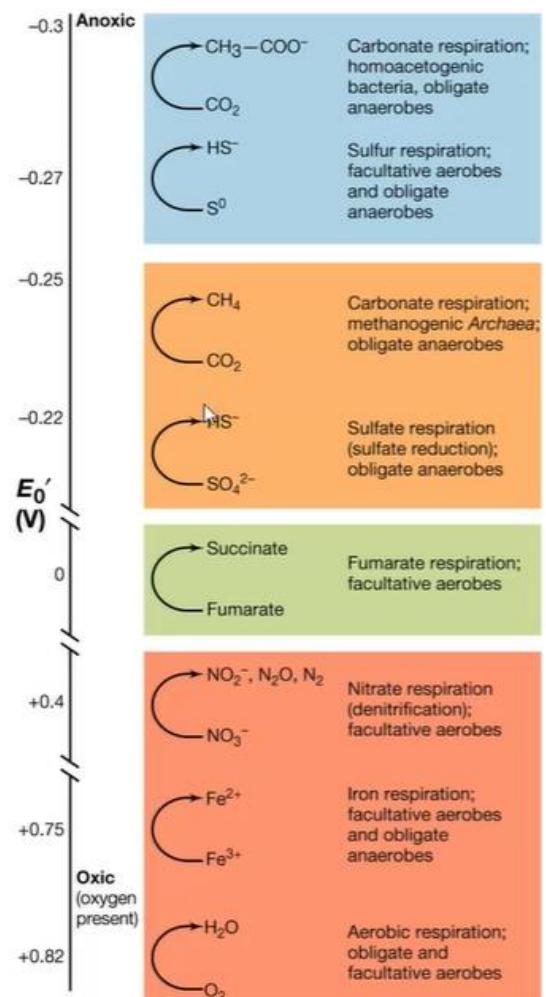


Usually, when respiratory chain is working well, all of the O_2 are reduced to H_2O but, considering that e-s are transported and the flux of e-s is from several respiratory complexes and carriers, sometimes e-s can skip on the respiratory chain complexes and O_2 can be reduced to form, for example, superoxide or other oxygen radicals. They are very dangerous for the cells because they immediately oxidized proteins, which are degraded and so they will not work, and lipids, which are peroxidised and this alters the structures of membranes. In mitochondria and cytosol, there are several enzymes that can eliminate and detoxify the radical oxygen species produced. These enzymes are for example **superoxide dismutase**, **catalase**, which are the most important. They can scavenge the oxygen species and protects our cells from oxide damage.

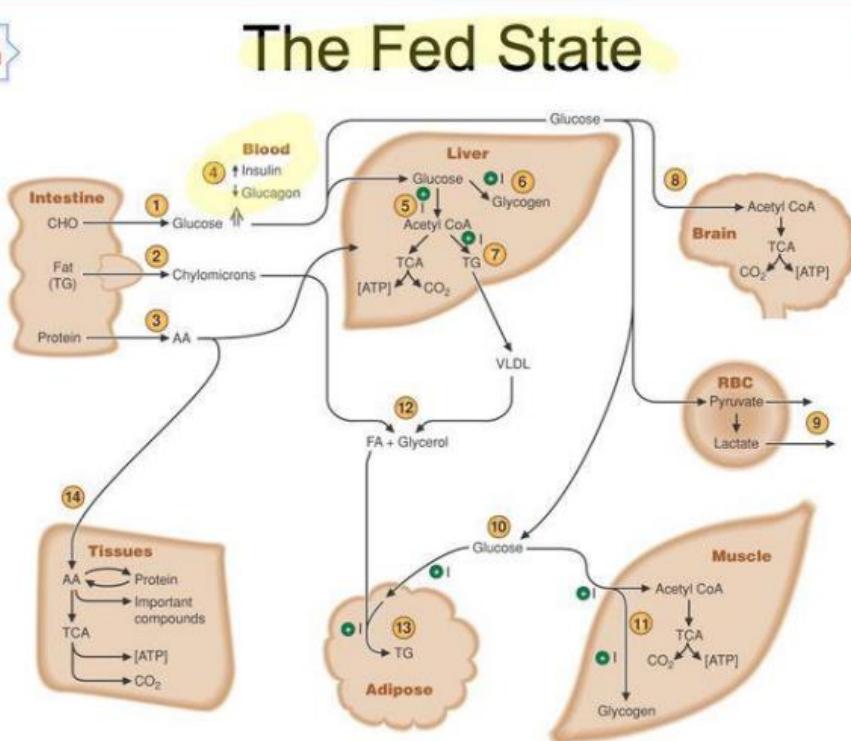
There are different e-s transport chain in bacteria that works more or less in the same way.

The electron donors are always NADH and FADH_2 but depending on the organism, the organisms can have different e-s transport chain that transport e-s from NADH to FADH_2 to another acceptor, which in anaerobic conditions is generally an inorganic molecules, such as sulphur, CO_2 , ..

The general concept is: if we have a membrane impermeable to p+s, if we have e-s carriers (like NADH and FADH_2), e-s transporter (like several enzymes that are very similar to the mitochondria enzymes in the respiratory chain), they can transport e-s to a final acceptor. If the final acceptor is O_2 we are talking about respiration otherwise we are talking about e-s transfer chain, which can use the energy deriving from redox to produce p+ gradient and the ATP synthase can use it to synthesis ATP.



What happens in our body in Fed State



When we are in fed state, the concentration of glucose in blood is increasing. So, insulin is produced which induces the uptake of glucose by all the organs but in particular by liver. If glucose is present at high concentrations, liver can use it for storing it (activation glycogen synthesis) and for the production of ATP for the other anabolic pathways. So, when we are in fed state, in liver glycolysis is activated to produce acetyl-CoA, which can be used for the synthesis of fatty acids and to produce ATP. Fatty acids are then transported as lipoproteins into the adipose tissue.

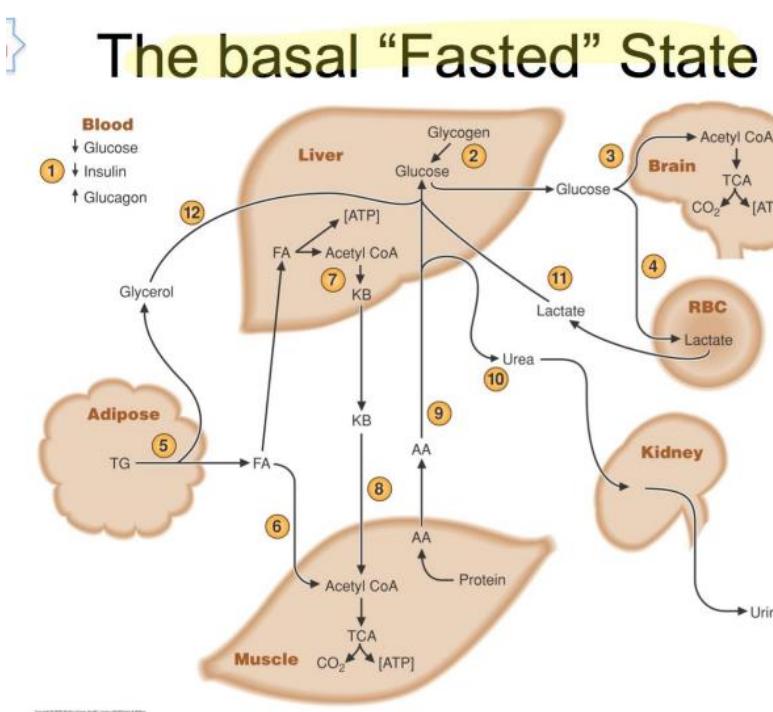
Inside it, they are used for the synthesis of triacylglycerol (TG) stored in the adipose tissue.

When we have high concentrations of glucose, brain can use it for producing energy (aerobic glycolysis). Instead, in blood cells, which use glucose because they do not have mitochondria, use glucose only in anaerobic way; from pyruvate, lactate is synthetized and after it can be transported in liver (gluconeogenesis).

Muscle can uptake glucose. In this case, muscle is able to activate gluconeogenesis and a part of glucose is used for the synthesis of ATP in aerobic glycolysis.

In fasted state you have the opposite conditions: concentration of glucose in the blood is increasing so glucagon is synthetised in pancreatic cells. Glucagon acts on liver to activate glycogenolysis to activate gluconeogenesis and to produce glucose that is released in blood. And again glucose is used in brain in an oxidative way to produce ATP, by red blood cells again in anaerobic way to produce lactate, which is transported in liver for the synthesis of glucose in gluconeogenesis.

In fasted state, in liver, we have also the oxidation of fatty acids in fact glucagon activates the adipose tissue to degrade TG to form fatty acids and glycerol, which can be phosphorylated into glycerol 3P and isomerised into the hydroxy acetone



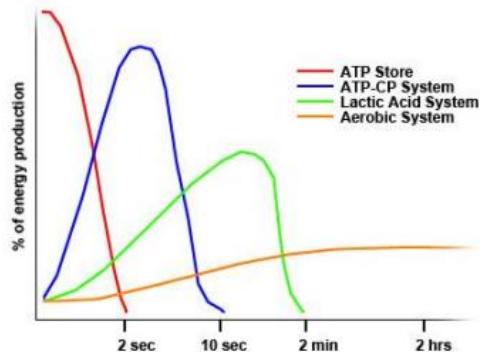
phosphate to enter in gluconeogenesis. On the contrary, fatty acids are oxidized in liver to form acetyl CoA and a part of NADH and FADH₂ synthesised the oxidation can be used to produce ATP in oxidative phosphorylation. But when the rate of fatty acids oxidation is very high and so very active, we have an accumulation of acetyl CoA and part of it is condensed to form ketone bodies, which are released in blood and used in particular by muscles, like heart. Ketone bodies, in muscle, are reconverted in acetyl CoA, which can enter in Krebs cycle and produce ATP by oxidative phosphorylation.

At the end, we have also the degradation of proteins into amino acids in muscles, but also in liver. Amino acids are deaminated and their carbon skeleton can enter in liver to be used for gluconeogenesis or in muscles they are directly oxidized in muscles to produce energy.

SOURCES OF ENERGY FOR MUSCLE CONTRACTION

Rate of ATP Synthesis is Dependent upon Substrate

Substrate	Relative Rate	
Phosphocreatine	100	Direct ~P transfer, no substrate oxidation required.
Glycogen (anaerobic glycolysis)	55	Phosphorolysis, G-1-P formed without use of ATP yielding 3 ATP/glucosyl group, "tree-structure" gives many simultaneous reaction sites.
Glucose (aerobic glycolysis)	23	Limited by rates of membrane glucose transport, glycolysis and mitochondrial oxidation.
Fatty Acids	10	Lipolysis only at interface between oil droplet and cytosol, limited by transport and mitochondrial oxidation.



Useful for giving us an idea how energetic molecules are used during sport.

Red: we have an amount of ATP that can be right away used for activity (firsts 2 sec)

Blue: phosphocreatine, a molecule present in our muscles and with an high energetic level. In the firsts 10 sec can be dephosphorylated and this phosphate group is used to produce directly at level substrate new ATPs. The amount of energy that this molecule can give us is essential for 10 sec of activity.

Green: energy is given by phosphocreatine but also by anaerobic degradation of glucose (glycolysis without mitochondria).

Orange: energy derives from aerobic glycolysis but in particular by the oxidation of fatty acids (after about 10 min). So, these two reactions are coupled.

16-Photosynthesis

The most important thing is that the photosynthetic organisms are able to acquire energy from the sunlight. This energy is converted into chemical energy, which corresponds to the synthesis of carbohydrates (remember that from them you can synthetise all the organic molecules).

THE PHOTOSYSTEM

Photosynthesis is the metabolic pathway present in photosynthetic organisms, they are able to acquire energy from the sunlight and convert it into chemical energy which correspond to the synthesis of carbohydrates. Photosynthesis occurs in protein complexes called **photosystems**. They have specific cofactors called **chlorophyll a** and **b** or other molecules (pigments called **light harvesting or antenna molecules**) that can acquire energy from the sunlight.

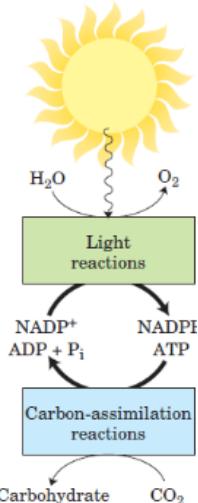
The chlorophylls are able to converge all the energy from the sunlight and transport it in an electron chemical energy that is transformed into chemical energy.

Pigments can absorb light energy and transmit it rapidly and efficiently to the reaction centre. They can acquire and converge the energy to the chlorophyll a and after it starts the first reaction in photosynthesis.

A photosystem is a protein complex in which in the central part we have the chlorophyll a and other components called primary e-s acceptors (can acquire e-s from chl. a) but the central part is called reaction centre (we have the real reactions). Around the reaction centre we have other proteins in which we have the other pigments: light harvesting can absorb energy and transport it to the chlorophyll.

The photosystems are embedded into the thylakoid membrane in plants and in the plasma membrane in bacteria.

In algae, cyanobacteria and vascular plants it is possible to divide photosynthesis in two:



• Light-driven reactions

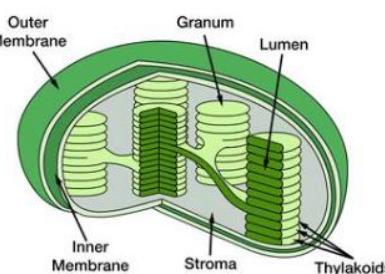
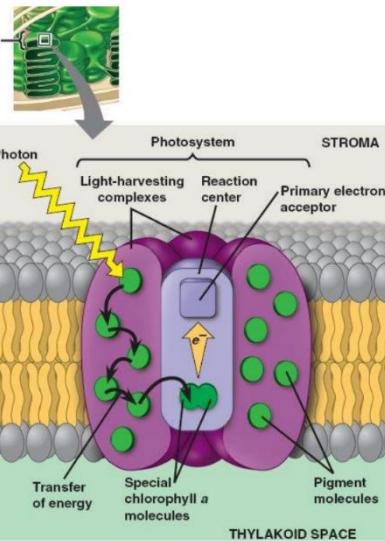
The sunlight energy is directly involved in the synthesis of ATP and NADPH, it is the opposite of respiration. Thanks to the energy, H_2O is oxidized to O_2 and the electrons deriving from it are transported through an electron chain that is used to reduce $NADP^+$ to NADPH. It is constituted by an endergonic reaction.

These reactions are connected to the next ones by electron chain transport. During the transport of electrons, there's a part of the chain in which the redox reactions are used for the formation of a proton gradient for the formation of ATP.

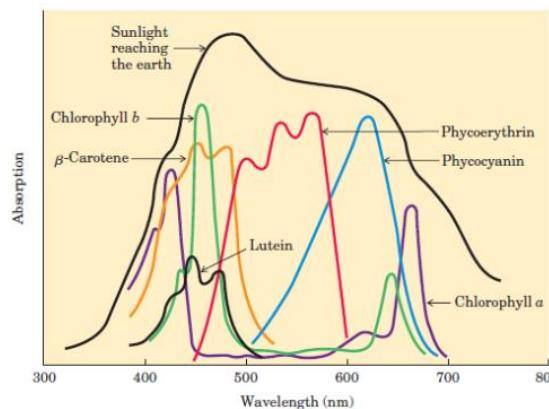
• Carbon-assimilation reactions

Occur during the day depending on light and they allow CO_2 fixation to form glucose. They use the ATP and NADPH produced by the light-driven reactions.

Chloroplasts share some similarities with mitochondria because they're formed by a permeable outer membrane, in addition they have an inner one. A membrane system is present inside the chloroplast formed by many flattened, membrane-surrounded vesicles or sacs (**thylakoids**), arranged in stacks which form the **grana**. Grana are surrounded by the **stroma** which contains most of the enzymes required for the carbon assimilation reactions. Therefore, there are 3 systems of membranes and 3 compartments: inner chloroplast space, stroma and grana.



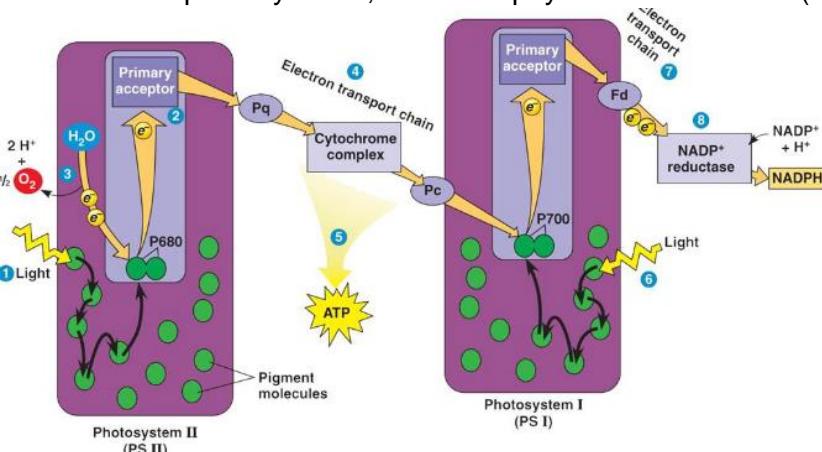
Chlorophyll A and B are both contained in the chloroplast and they're the most important light-absorbing pigments in the thylakoid membranes. **Accessory pigments** are present in light-harvesting complexes or antenna complexes, they can absorb the energy in the spectrum of visible light.



Chlorophyll A can absorb the energy in the wavelengths between 650-700 nm, and the antenna complex is needed for the absorption of the energy from all the visible spectre that then is converted to the chlorophyll A by resonance.

The photosynthetic apparatus of modern cyanobacteria, algae, and vascular plants, there are two photosystems:

In photosystem I, the chlorophyll A is called P700 (700 nm is the peak of maximum absorption).



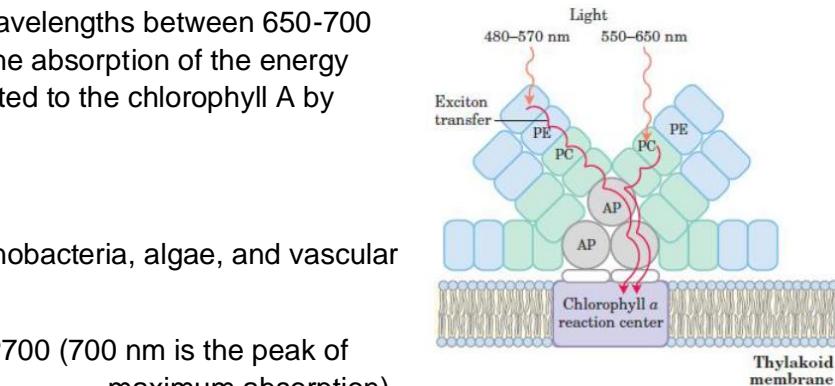
same as coenzyme Q.

Plastoquinone can diffuse in the thylakoid membrane and can react with an enzymatic complex very similar to complex 3 (respiratory chain complex which can oxidize it and reduce cytochrome C). In plants this complex is called B6F complex. Cytochrome B, F is for the iron sulphur protein present inside the complex.

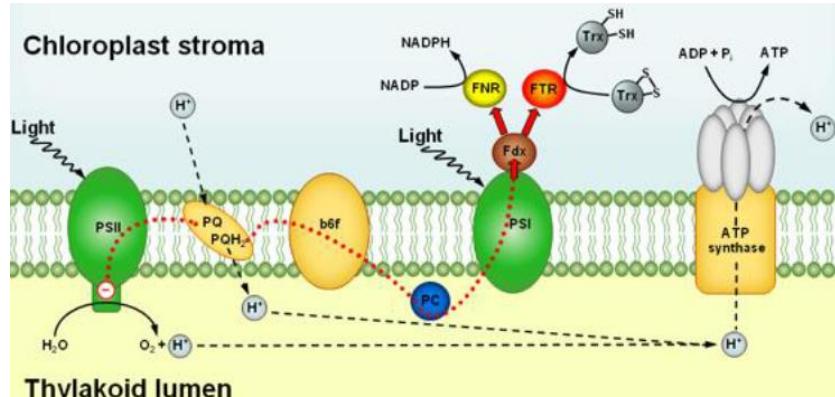
The other acceptor is called **plastocyanin**, similar to cytochrome C, is a soluble protein present inside the lumen of thylakoids, it doesn't have any heem groups but it can transport electrons thanks to a copper ion that can be reduced and oxidized.

PS II \rightarrow Pq \rightarrow Cytochrome complex (takes electrons from Pq and reduces Pc) \rightarrow Pc

Photosystem I is also inserted in the thylakoid's membrane, with a P700 in the reaction centre. Light can activate the chlorophyll A and then the electron is extracted and brought to the primary acceptor. Then the primary acceptor can give electrons to **ferredoxin**, which is an electron transporter, that brings the electrons to **NADP⁺ reductase** that can reduce NADP⁺ to NADPH.



In photosystem II, the chlorophyll A is called P680. When this second is excited, it loses an electron which is transferred to the primary acceptor which can give it to a soluble electron transporter (lipophilic), which is very similar to ubiquinone, and it is called **plastoquinone** (Pq). It has a head that can be reduced (acquire 2 e), it has an isoprenoid chain with different number of isoprenic units but the chemical characteristics of plastoquinone are the same as coenzyme Q.



During the excitation of chlorophyll, since it loses an electron, to undergo a second reaction it needs to acquire another electron: form water in case of photosystem II, and plastocyanin for photosystem I.

It's also worth noticing that the process starts from a molecule which is able to give two electrons, and then it finishes with the reduction of NADPH that requires two electrons.

This whole process creates a proton gradient that gets dissipated by ATP synthase to synthetise ATP. The results form the whole process is NADPH and ATP.

Photosynthesis process gets regulated by enzymes that are active in presence of light thanks to **thioredoxin** (Trx – grey in the picture) that in its reduced form it can react with photosynthesis' enzymes activating them. Therefore, during the day thioredoxin is reduced because part of electrons used in photosynthesis are used to keep thioredoxin with two sulfhydryl group. During the night thioredoxin is oxidized, inhibiting the enzymes involved in carbonisation of CO₂.

The distribution of PSI, PSII and ATP synthase is specific in thylakoids membrane.

- PSI is present prevalently in membrane thylakoids towards the stroma because of the need to react with ferredoxin.
- ATP synthase is also towards the stroma in order to reach to ADP and phosphate.
- PSII present thylakoids membranes which are separated from the stroma since it needs water and plastoquinone.

Photosynthesis machineries in bacteria are of many kind.

In purple bacteria, the photosystem centre can be excited, so the chlorophyll can carry the excited electron which has a very negative redox potential. So that the electron can reach the electron transporters like quinone.

Can be released to electron transporters like quinone that can be reduced by our photosystem, for example in bacteria the primary acceptor is the molecule called priendic that can after give electrons to quinone, which after reduction it can be oxidized by a complex similar to complex 3

In case of the purple bacteria, the electron donor to the chlorophyll after excitation is cytochrome C2 and here a cyclic serie of reaction brigs no production of NADPH but there's the formation of the proton gradient, then, thanks to the activity of the BC1 complex, ATP gets synthetised.

In green sulphur bacteria, which are more evolved, there's the presence of ferredoxin which allows the synthesis of NADH.

Calvin cycle

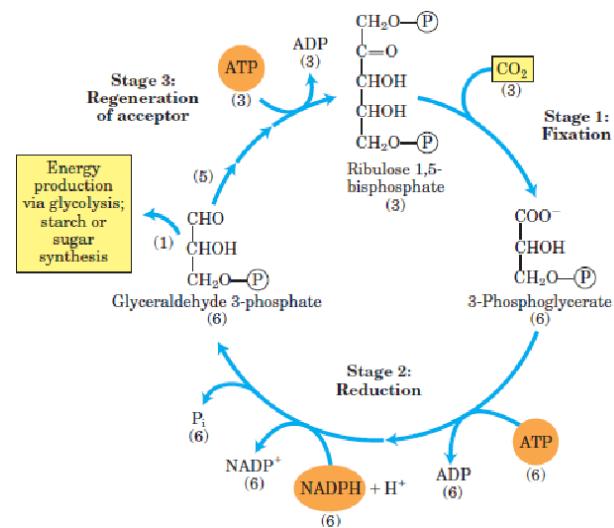
All the reactions are light dependent, but not light driven. ATP and NADPH are used to carry on the CO₂ assimilation in a cyclic process named Kalvin Cycle.

The cycle can be divided in 3 phases and for each stage we consider that the first reaction occurs three times:

Fixation Three carbon dioxide molecules get assimilated by condensation with three ribulose 1,5-biphosphate forming three 3-phosphoglycerate. The reaction is catalysed by rubisco which is an enzyme present in all the photosynthetic organisms.

Reduction 3-phosphoglycerate is reduced using ATP forming 1,3-biphoshoglycerate, NADPH is used to dephosphorylate and reduce again the resulting molecules to give glyceraldehyde 3-phosphate. At the end of this second part, we have the formation of 6 molecules of glyceraldehyde 3-phosphate. The reaction requires 6 molecules of ATP and 6 of NADPH, one for each reduction, the phosphates freed are also 6.

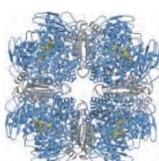
Regeneration of acceptor One molecule of glyceraldehyde 3-phosphate exits and goes to the gluconeogenesis pathway to be used for sugars, fatty acids, or amino acids. The 5 molecules left



gets reorganized in order to form again 3 molecules of ribulose 1,5-biphosphate thanks to the loss of two phosphates and hydrolysing 3 ATP.

For the organization of three molecules of CO₂ to produce a molecule of glyceraldehyde 3-phosphate which is the molecule that can enter gluconeogenesis, are required 9 ATP and 6 NADPH. Very energy consuming process.

➤ First stage

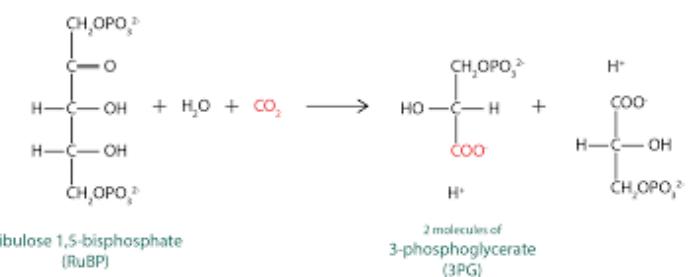


First reaction of the Calvin Cycle is the incorporation of CO₂ into an organic form, and it is catalysed by rubisco which is the short name for ribulose 1,5-bisphosphate carboxylase/oxygenase.

Rubisco has as substrate ribulose 1,5-bisphosphate and carbon dioxide, it is an allosteric enzyme formed by 4 subunits and in some cases can reach 8 subunits. In each subunit there's a reaction of compensation.

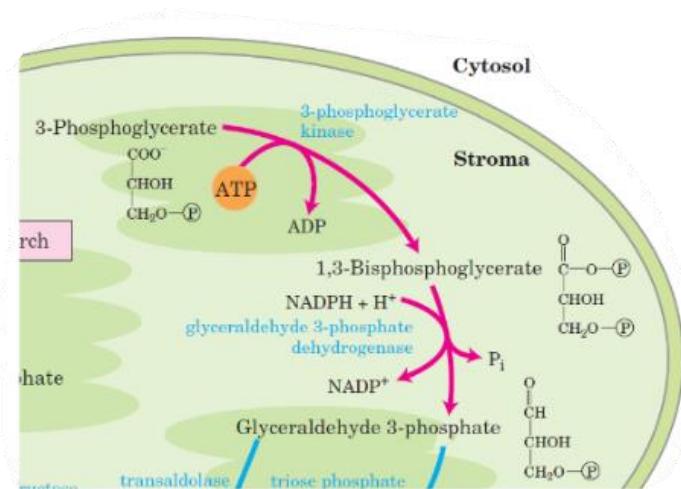
During the reaction, the intermediate of reaction is very unstable and it is instantly broken down into two molecules of 3-phosphoglycerate (3PG).

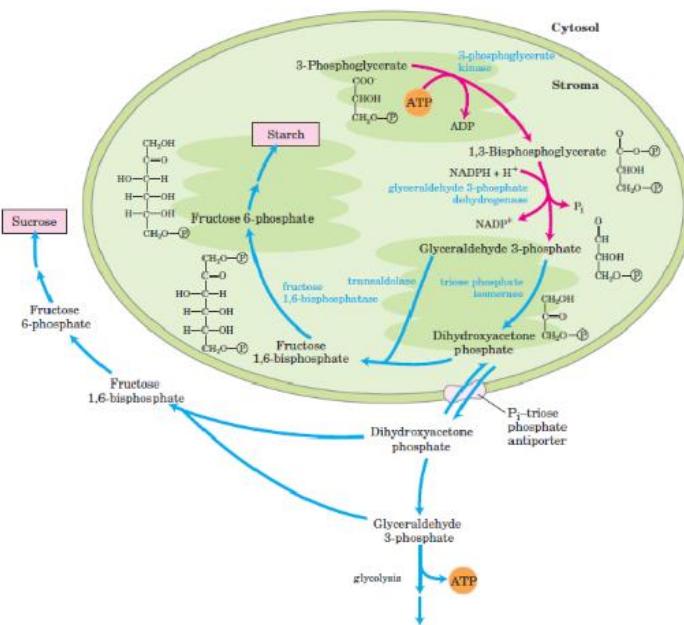
Rubisco's synthesis occurs in chloroplast (they have DNA like mitochondria) and its activity is strictly regulated by light. This enzyme has a slow rate of reaction but since it is very abundant it does not limit the velocity of the reaction. It is also called oxygenase because it can also react with oxygen, this is because when it had been first synthesized, CO₂ was the most abundant gas in the atmosphere, so rubisco didn't need to be specific for one or the other.



➤ Second stage

In the stroma of the chloroplast there are the enzymes which catalyse the first reaction of gluconeogenesis: 3-phosphoglycerate is phosphorylated to form 1,3-biphosphoglycerate with the use of a molecule of ATP (6) for each reaction. 1,3-biphosphoglycerate is then reduced (still gluconeogenesis reaction) to form glyceraldehyde 3-phosphate using NADPH. ATP and NADPH are the product of light reactions in photosynthesis.





➤ Third stage

From the 6 glyceraldehyde 3-phosphate molecules, one gets isomerized into dihydroxyacetone phosphate so the reaction of gluconeogenesis can occur (condensation with 3-phophoglyceraldehyde to form fructose 1,6-bisphopshate which is then dephosphorylate to fructose 6-phosphate which is isomerized into glucose 6-phosphate that is then turned in starch).

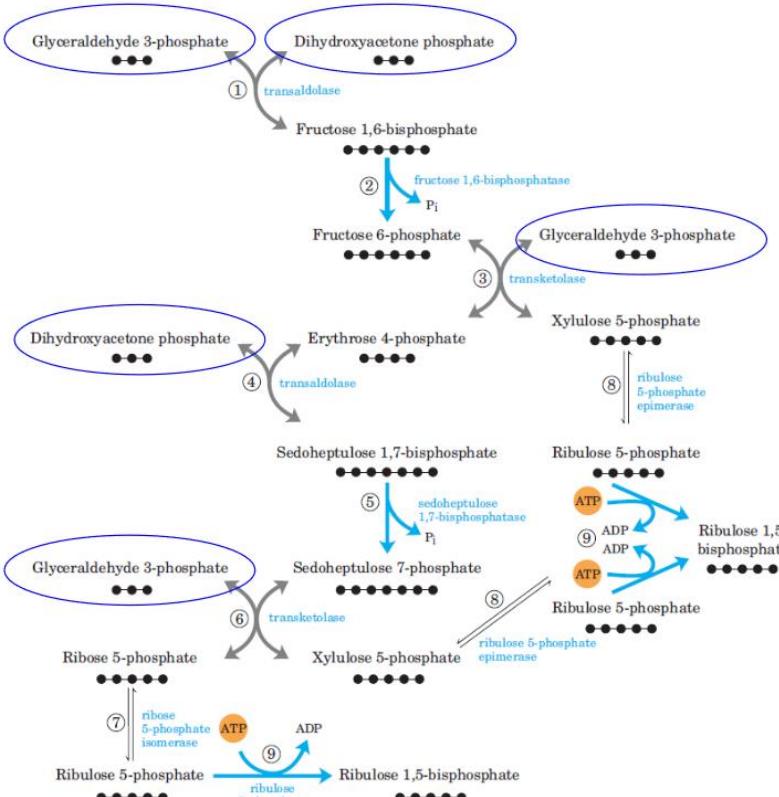
During the day starch is temporarily stored in chloroplasts, then during the night starch is degraded into glucose and fructose to give sucrose which is released from the chloroplast and transported along the plant to the storage organs, here glucose and fructose are reformed so that they can be used for storage and starch.

The five molecules of glyceraldehyde 3-phosphate are reorganized to regenerate ribulose 1,5-biphosphate, some of these reactions are similar to the reactions of pentose phosphate pathway.

Glyceraldehyde 3-phosphate can be isomerized to dihydroxyacetone phosphate by the isomerase present in glycolysis and gluconeogenesis. So, the first two molecules of glyceraldehyde 3 phosphate can be condensed as a molecule of dihydroxyacetone phosphate to form fructose 1,6-bisphosphate. Fructose 1,6-bisphosphate is dephosphorylated into fructose 6-phosphate (reaction of gluconeogenesis), so then it can react with glyceraldehyde 3-phosphate thanks to transketolase (enzyme of the pentose phosphate pathway) forming erythrose 4-phosphate and xylulose 5-phosphate (intermediates of PPP).

Xylulose is epimerized to form ribulose 5-phosphate that can be phosphorylated to form ribulose 1,5-bisphosphate (new reaction specific for Calvin cycle), so we get the first molecule able to undergo again the Calvin cycle.

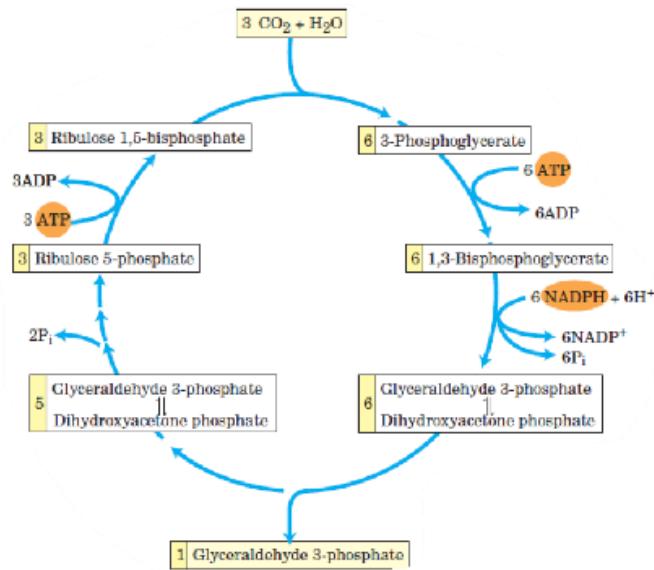
Erythrose 4-phosphate can condense with dihydroxyacetone phosphate by transaldolase (enzyme in PPP) forming sedoheptulose 1,7-bisphosphate. This molecule is then dephosphorylated to get sedoheptulose 7-phosphate. It is possible then for this molecule to react with glyceraldehyde 3-phosphate by mediation of transketolase which gets ribose 5-phosphate and xylulose 5-phosphate. The second is epimerized to ribulose 1,5-bisphosphate and the first instead is isomerized into ribulose 5-phosphate and then phosphorylated.



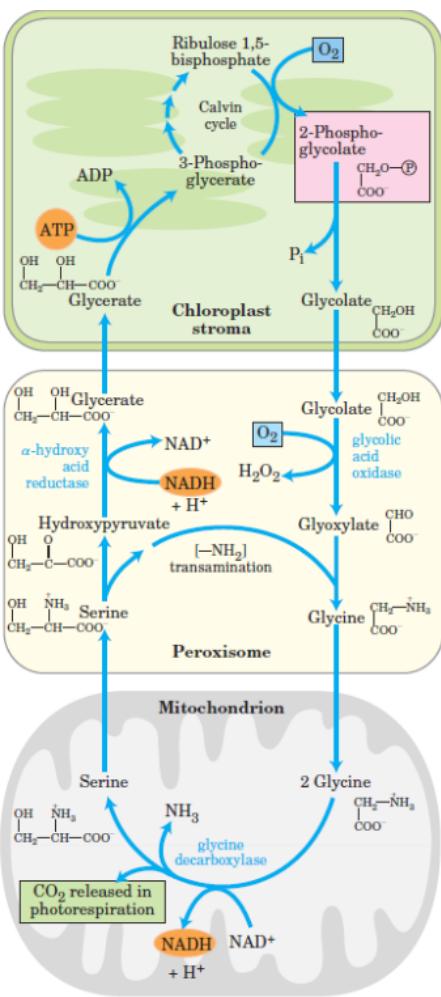
There's no need to remember all the reaction but she could show the image without name and ask you to comment the reactions, important to know:

- which reactions are similar to the PPP so that she gets convinced that we also studied that;
- how many are the molecules of glyceraldehyde 3-phosphate required for the synthesis of 3 molecules of ribulose 1,5-bisphosphate;
- which are the exergonic reactions in this pathway (blue arrows), they make the whole pathway irreversible.
- For the synthesis of a mol of glyceraldehyde 3-phosphate, 3 mol of CO₂ are assimilated, 9 mol of ATP are required and 6 of NADPH required for reductive reactions.

What happens when rubisco reacts with oxygen and not CO₂?



Photorespiration / oxidative photosynthetic carbon cycle / C2 cycle



Rubisco is not specific for CO₂ so once in every three or four turnovers uses O₂ forming a useless compound called 2-phosphoglycolate and a molecule of 3-phosphoglycerate which can go on in the cycle. The cell then needs to recycle 2-phosphoglycolate in a quite complicated pathway, the reactions occur in three different cell compartments, from chloroplasts to peroxisomes and then mitochondria. For plants these reactions are important because this molecule is toxic and cannot be accumulated.

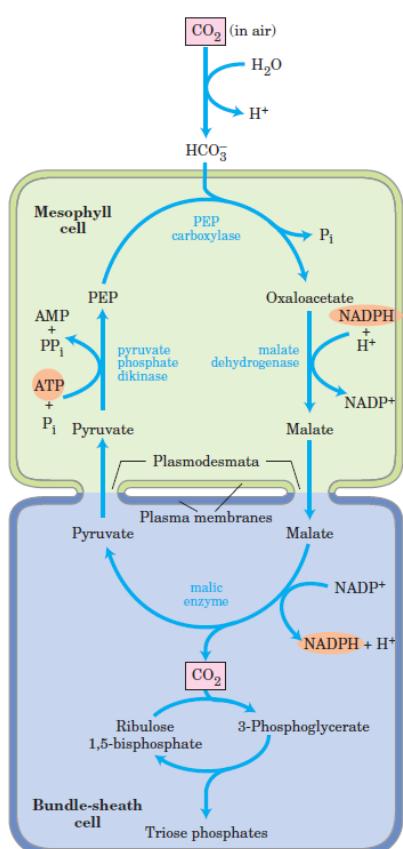
- Chloroplast stroma : 2-phosphoglycolate is dephosphorylated into glycolate
- Peroxisomes : Glycolate is oxidized by an oxidase where O₂ is reduced to hydrogen peroxide. Peroxisomes are organelles in which hydrogen peroxide can be synthesized and the detoxifying enzyme like catalase can degrade it into water. Furthermore, in peroxisome, hydrogen peroxide can be used as a defence mechanism to kill bacteria. Glyoxylate is the result of the glycolate oxidation, this product is aminated to synthetise glycine, the amino group derives from serine through a transaminase.
- Mitochondria : Two molecules of glycine are oxidized, deaminated, lose a molecule of CO₂ to finally synthetise serine. Serine now is then transferred to peroxisomes.
- Back to peroxisomes : Serine can give the amino group to glyoxylate, and its carbon skeleton can be reduced to form glycerate which is phosphorylated to form 3-phosphoglycerate
- Back to chloroplast : Glycerate is phosphorylated to form 3-phosphoglycerate that is able to re-enter the Calvin cycle.

There are a lot of reactions to reorganize the 2-phosphoglycolate to form the 3-phosphoglycerate to re-enter the Calvin cycle. It is a quite expensive cycle because of the use of ATP and the transport between compartments.

The characteristic of this cycle is that during the cycle there's the consumption of O₂ and production of CO₂.

Photosynthesis is more efficient than photorespiration so the energy deriving from photosynthesis can be also spent for eliminating toxic compounds with photorespiration. This is not true in hot and arid climate, since photorespiration is particularly active when it's very warm, the plants which live in arid areas do not open the stomata in order to not lose water. This leads to a higher concentration of O₂ in chloroplast that use it instead of CO₂.

The solution is storing CO₂ in chloroplasts so that they don't need to open the stomata still avoiding photorespiration. There are two types of mechanisms that these plants use: C4 plants and CAM plants.

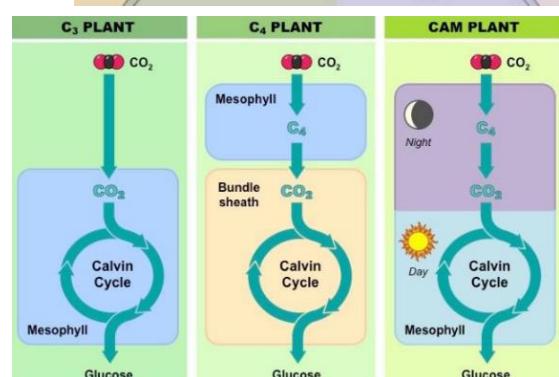
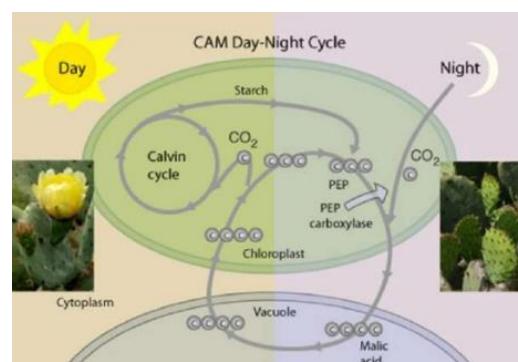


C4 plants can concentrate CO₂ inside the chloroplast, for example corn. These organisms have a different anatomy than C3, there's both mesophyll cell and the bundle cell in which the chloroplasts are active for photosynthesis. When the stomata is open, CO₂ can enter, transported inside the mesophyll cell and it reacts to form malate (4 carbon molecule) which is then accumulated there. Under necessity, malate can be transferred to the bundle cell that is able to free CO₂ and undergo photosynthesis avoiding high level of photorespiration.

CO₂ inside mesophyll cells react with phosphoenolpyruvate which is carboxylate to form oxaloacetate. The enzyme catalysing this reaction is PEP carboxylase which we already saw in the anaplerotic reactions. Oxalacetate is reduced in malate which is accumulated and eventually transported in the chloroplasts of the bundle cells. Here it is oxidized and decarboxylated to form pyruvate and CO₂.

CAM plants are instead cactus and succulent plants. Here the synthesis of malate happens during the night.

The CO₂ enters the mesophyll cell, during the night it is fixed to form malate (in the same way as C4). Malate is transported and concentrated inside the vacuole, during the day instead it is brought to the chloroplast to supply the Calvin cycle. In this case the separation is not due to the anatomy of the cells but is given by the time of the day (day or night).



C3 plants are the ones present in our areas, they have that name because the intermediates of reactions are formed by 3 C. Mesophyll cells have high concentration of chloroplasts and are the active cells for the synthesis of sugars. In these plants, the stomata is almost always open and the photorespiration happens not very frequently.

17-General workflows in metabolomics

How to prepare the sample, how to choose one and what is the workflow for a metabolomic experiment.

Principle of metabolomics

Metabolomics= systematic identification and/or quantification of wide ranges of small molecule metabolites in bio-samples.

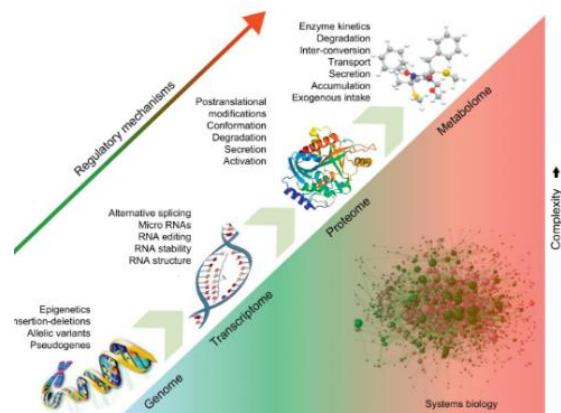
Metabolome= all low molecular mass compounds synthetized and modified by a living cell or organism. Includes the endometabolome (metabolites present inside the sample) and the exometabolome (metabolites secreted by the metabolomical sample).

Metabolomic fingerprinting= spectra from NMR or MS analysis that provides a fingerprint of metabolites produced by a cell (endometabolome). Rapid and general screening.

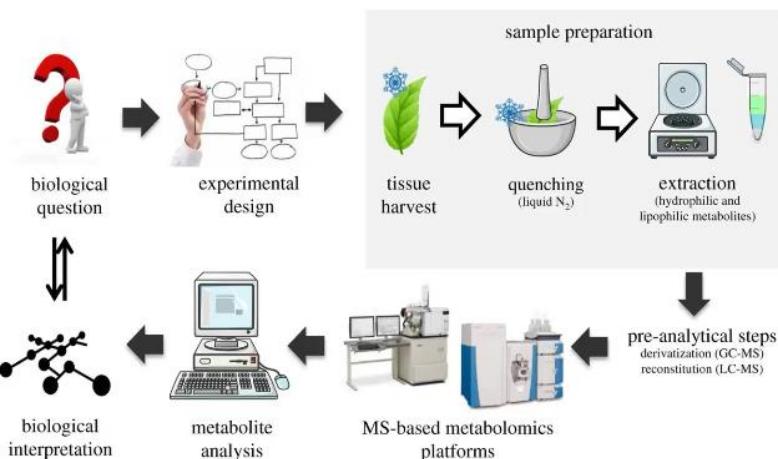
Metabolomic footprint=analysis of the exometabolome, either through analysis of metabolites or through spectra that do not provide information about specific metabolites.

Metabolic profiling= analysis of a pre-defined set of metabolites, usually part of particular metabolic pathway. Not necessarily quantitative but at least semi-quantitative. Oldest and most established approach. The preparation of the sample is different because we want to quantify and detect specific metabolites.

Metabolomics is the youngest omics technique, and it tries to put together all the information from the other omics sciences in order to reach system biology. Metabolomics is difficult because it requires a study of many different molecules with different size, weight, polarity, stability, concentration, they're compartmentalized, and their identification is quite difficult.



General workflow



Depending on the biological question it is possible to develop experimental design. After choosing the best one, the next step is preparing the sample: sample selection; quenching means to make all the reactions stop so that the metabolites are fixed in their concentration and composition; extraction works on the different chemical and physical characteristics of the metabolites.

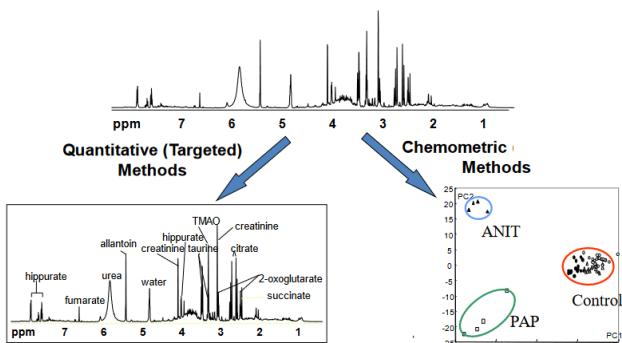
Most used analytical methods are Nuclear Magnetic Resonance and Mass Spectrometry, they have some specific characteristics.

Now from the list of all the metabolites, we have to analyse them and check whether the biological question had been answered.

Biological question “the science is in the question, the answer is in the experimental design”

Experimental design

- Targeted metabolomics = a quantitative analysis (concentrations are determined) of a few metabolites and/or substrates of metabolic reactions that might be associated to common chemical classes or linked to selected metabolic pathways (ex. metabolic profiling)
- Untargeted metabolomics = a qualitative analysis of the largest possible number of metabolites contained in a biological specimen (ex. fingerprinting and footprinting)



Quantitative (targeted) methods gives you the name of specific metabolites which are of interest

Chemometric (untargeted) methods allows instead to group metabolites to understand the composition of metabolites in our biological sample.

During the experimental design its needed to take in consideration the sample type (biological fluids, tissues, cells, intact organisms, ...), the sample size (number of specimens to be assessed), and the experimental conditions (treated/untreated samples, frequency of sample collection etc...)

Sample preparation

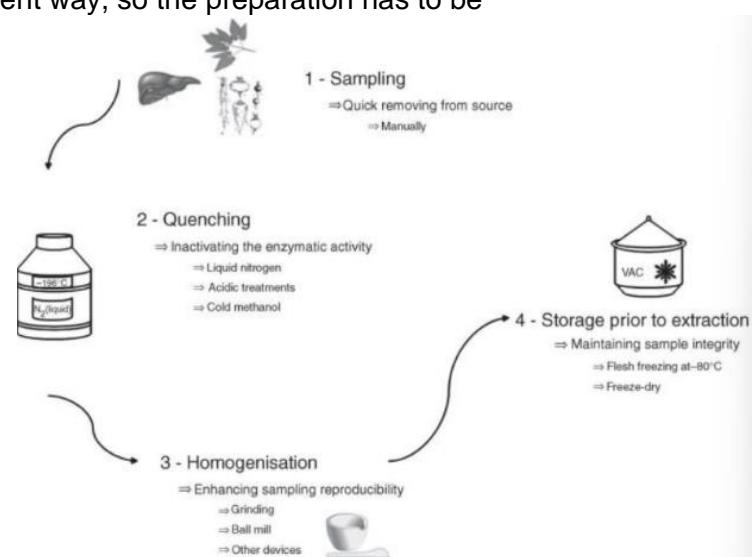
Its very important and it is related to several characteristics of our experimental design:

- Sample type (cell, tissue, biological fluid), so check whether there's the presence of a cell wall that needs to be disrupted or something like that.
- Selection of metabolomics approach (targeted vs untargeted):
 - For targeted its needed to undergo optimized procedures in order to detect better the specific metabolites of interest
 - For untargeted extraction is done by solvent considering the different characteristics (extract both hydrophobic and hydrophilic, etc.), in order to extract as many metabolites as possible.
- Analytical method because they work in different way, so the preparation has to be different.

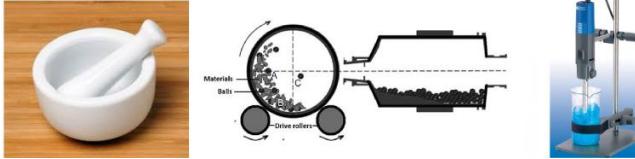
One of the most important steps is the quenching because the time and the way in which you do it, can influence a lot the sample.

It is also fundamental the homogenization so that we are able to extract better our metabolites, it has to be performed very carefully.

There are many quenching methods, and they depend on the metabolites and the type of metabolomic experiment:



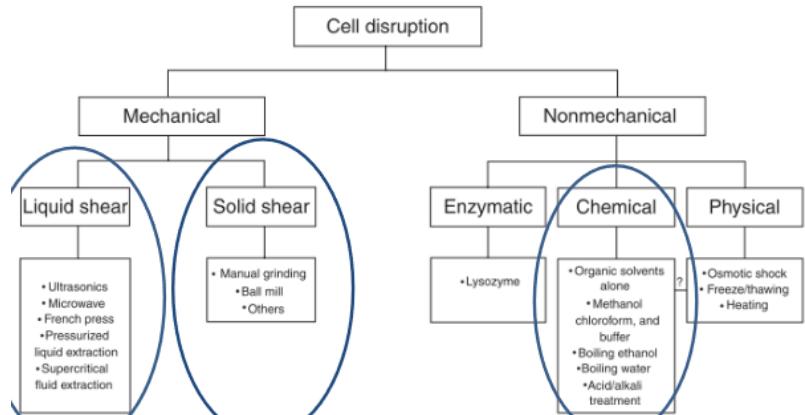
For example, if we want to analyse the exametabolome, it is possible to undergo filtration or centrifugation. Instead, for fingerprinting or to detect the endometabolome, we need to stop the activity of all the enzyme to have a picture of the metabolomes in a specific time, this can be done in cold conditions (-180°C), or in hot conditions so that the enzyme denature, or using acidic or basic conditions because each enzyme has a specific temperature and pH in which is able to work.



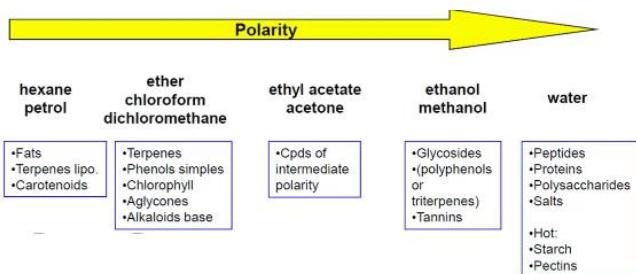
homogenization is done using the ultraturex (? , not imp anyway) which works as a mini pimer.

Metabolite extraction aims to disrupt the cell structures liberating all or the maximum number of metabolites in their original state and in a quantitative manner to a defined extraction medium. There are basically two methods: mechanical and nonmechanical. Furthermore there might be the need to target only some specific metabolites so the extraction has to be targeted.

Homogenization and storage are also different depending on the sample. The goal is to disrupt our biological sample. Homogenization most of the time allows quenching at the same time. In case of the presence of tissues deriving from muscle or other fibrous organs, the



There is no single procedure for metabolome analysis that extracts all intracellular metabolites. This is due to the fact that there are many metabolites with very different characteristics, so its not possible to extract them all.



Organic solvents with different polarities are widely used for extraction of intracellular metabolites.

Sometimes it is possible to perform different steps of extraction, for example starting from a polar solvent:

after solubilizing with polar solvent many times, it is possible to separate the precipitate and the different phases to differentiate from the most solvent to the least. Anyway, this process cannot make sure that no metabolite had been discarded.

Detection techniques

The two most used techniques are:

NMR SPECTROSCOPY	MASS SPECTROMETRY
<ul style="list-style-type: none"> Less sensitive for metabolite detection Non-destructive, requires little sample handling & preparation: <ul style="list-style-type: none"> Metabolites in liquid state (serum, urine, and so on) Intact tissues (es. tumors) or in vivo measurement Quantification easy: <ul style="list-style-type: none"> Peak area of compound in NMR spectrum directly related to concentration of specific nuclei (es. ^1H, ^{13}C), making quantification of compounds in complex mixture very precise 	<ul style="list-style-type: none"> More sensitive for metabolite detection <ul style="list-style-type: none"> Mass spectrometry can detect analytes routinely in femtomolar to attomolar range Require more tissue destruction Difficulty in quantification

Concentration detection:

NMR around molar and micromolar

MS up to picomolar

	NMR (with cold probe)	GC-MS (gas chromat. /mass spectrom.)	DI-MS (direct infusion mass spectrom.)
Techniques			
Metabolites	Water-soluble (amino acids, organic acids, sugars)	mainly water-soluble (some hydrophobic)	Mainly hydrophobic (some water-soluble)
Types of samples	Biofluids, plant, bacterial, animal tissue extracts, Food	Biofluids, plant, bacterial, animal tissue extracts, Food	Mainly biofluids
Sample Volume	100 μL (min)	30-50 μL (min)	10 μL
	NMR	GC-MS	DI-MS
Sample prep time	30 -120 min/20 samples	30 -120 min/20 samples	3-4 h for 96 samples
Run time	10-90 min/sample	30-60 min/sample	7 min/sample
Data Analysis	30-60 min / sample	30-60 min / sample	1-2 h for 96 samples
Limit of Detection	$\sim 5 \mu\text{M}$	$\sim 100 \text{nM}$	$\sim 5 \text{nM}$
No. of metabolites	$\sim 20-150$	$\sim 20-150$	$\sim 100-180$

In NMR water-soluble metabolites detection is limited to soluble metabolites, so a big part of the content can be lost.

18-Mass spectrometry

GENERAL ASPECTS

Mass spectrometry is one of the two techniques that perform metabolomics, give us the number that described molecules that are detected by metabolites.

Definition: is the art of measuring atoms and molecules to determine their molecule weight, we are going to analyse only ions. Ions correspond to molecules or fragment of molecules, we need molecules in ions form.

- **MASS SPECTROMETRY** is a sensitive analytical technique which is able to quantify known analytes and to identify unknown molecules at the picomoles or femtomoles level.

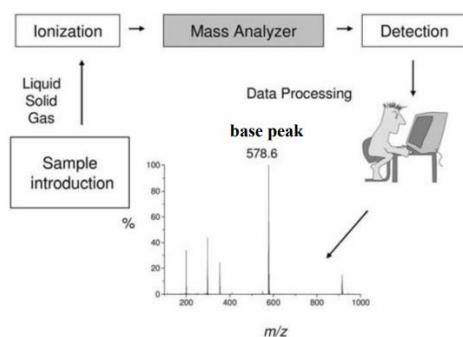
- A fundamental requirement is that atoms or molecules are ionized and analysed as gas phase ions which are characterized by their mass (m) and charge (z).

Molecules are in gas form and ions form.

- A **MASS SPECTROMETER** is an instrument which measures precisely the abundance of molecules which have been converted to ions., this techniques is able to know the mass of the molecule (that depends on the principle of separation of ions).

- In a mass spectrum **m/z** is used as the dimensionless quantity that is an independent variable.

- Most mass analyzers operate under high vacuum or at low pressure, so that the charged particles do not deviate from their trajectories due to collision with residual gas and thus never reach the detector



ionization: is the starting point for mass spectrometry

mass analyzer: able to separate the different mass to charge reaction

detection: signal in ions in flow of electrons that is record on our computer.

The graph is the output, we obtain the mass of different ions.

Analyte: the molecules we are interest in, the molecules

in a mixture that we want to detect and identify or only quantify if they are already know.

IONIZATION TECHNIQUES

- In the **ION SOURCE**, positive or negative ions are produced either under vacuum or at atmospheric pressure.
- Depending on the ionization technique either molecular ions (M^+) with an odd electron number or protonated (molecules already cation), deprotonated ions with an even electron number are formed. The molecule lost an electron, that's why is protonated.

$[M+H]^+$ → molecule + H is protonated molecules, charge is positive

$[M-H]^-$ → molecule -H, we have an ion, even electrons number, no loss of electrons, not radicals, this is the typical ionization for atmospheric pressure.

Sample introduction	Ionization Techniques	Mass Analyzer	Detector	Calculator
Direct introduction	El; Cl	Quadrupole	Ionic energy to electric signal	Data elaboration and storage
Direct infusion HPLC GC	ESI APCI MALDI	TOF Ion Trap Orbitrap		

DIFFERENT IONIZATION METHODS

- Electron Impact Ionization (EI - Hard method)

- Chemical Ionization (CI – Semi-hard)
 - Small molecules, 1-1000 Daltons, simple spectra

These 2 first ionization are made under vacuum.

- Electrospray Ionization (ESI - Soft) (atmospheric pressure)
 - Small molecules, peptides, proteins, up to 200,000 Daltons
 - Atmospheric Pressure Chemical Ionization (APCI - Soft)
 - Small molecules, up to 2000 Daltons
 - Matrix Assisted Laser Desorption (MALDI-Soft) under vacuum too
 - Smallish molecules, peptides, proteins, DNA, up to 500 kD

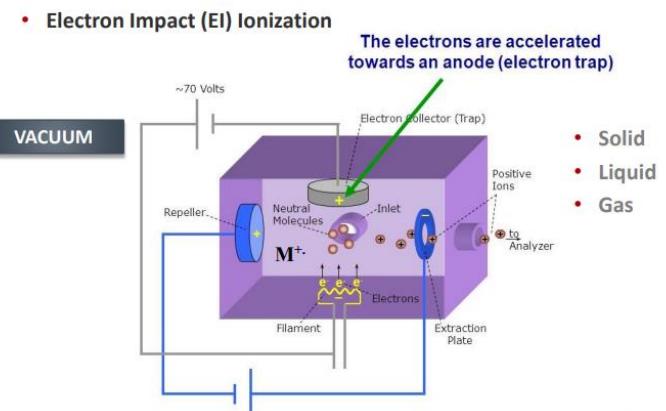
The most common ionization source are:

♣ ELETTRON IMPACT IONIZATION (EI)

(That works in vacuum)

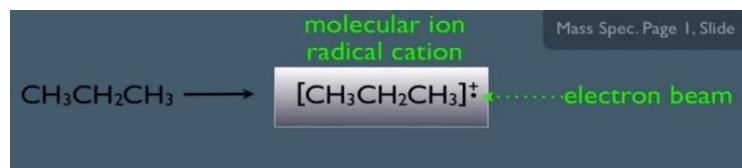
• ELECTRON SPRAY IONIZATION (ESI)

(Soft ionization source. Atmospheric pressure, combined with liquid chromatography)



PRINCIPLES OF IONIZATION

- If we find some substance and we don't know what it is, using mass spectrometry we can know the molecular mass of this molecule and other information about its possible structure.
 - In the mass spectrometer we shoot an electron beam at the molecule and the electron beam knock out an electron from the molecule.
 - It could happen that an ion of this molecule it would be created

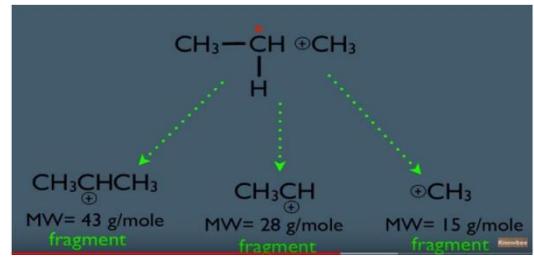


What we obtain firstly is the ionization of the molecule as a radical cation

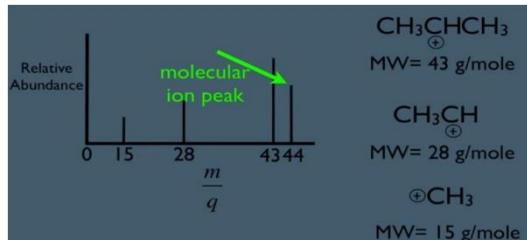
PRINCIPLES OF MASS SPECTROMETRY

- Propane can produce three kinds of fragments with different molecular weights
 - The electron beam could also break a bond inside the molecule creating a different ion with a different molecular weight called “fragment”

Here we have the fragmentation of the molecules, the original is a radical cation but then the fragments are simply cations



The peak corresponding to the heaviest MW is called molecular ion peak and usually correspond with the MW of intact molecule.



This is the mass spectrometry

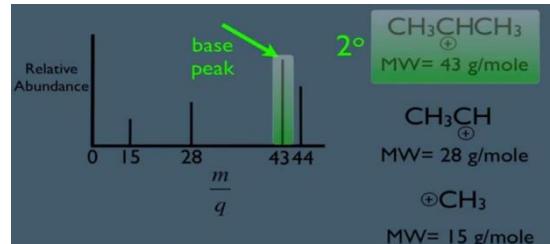
The other peaks are called fragment ion peaks and correspond to all the possible ion formed breaking covalent bonds

- The most abundant peak is called “base peak”. (green one)

- It is the most abundant cation that is also the most stable. In this case the cation in a secondary carbon.

- The complete fragmentation and rearrangement pattern is highly compound specific and is a powerful tool for identification of unknown compounds

- Now we have a great collection of a very large libraries of spectra that can be a great assistance for identification of unknown compounds

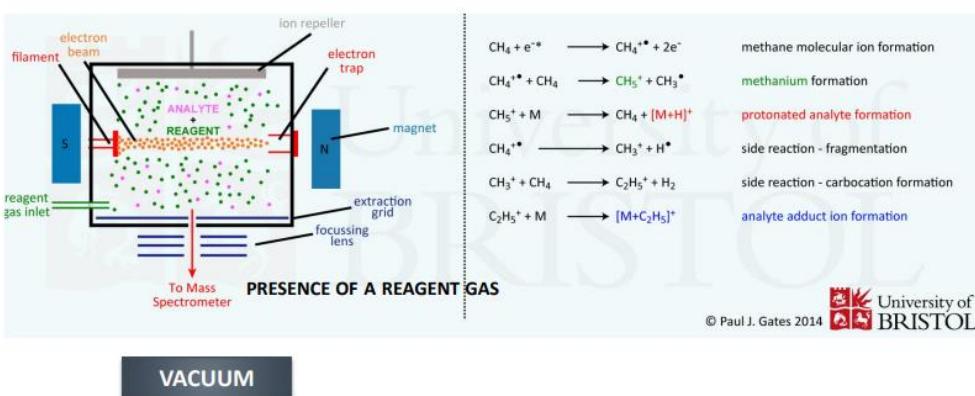


- If we know the molecules that we want detect, we can compare the spectra of each pure standard with the spectra of mixture and find if our molecule is present in mixture.

- If we don't know what are the molecules in the mixture, we can compare a pre compiled data base of known molecules spectra and find which molecules are present in the mixture.

Ionization techniques

- **Chemical Ionization**



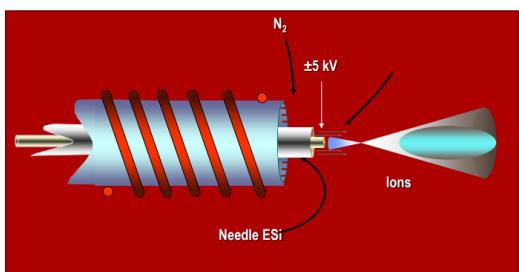
The chemical ionization is made in vacuum, it's a semisoft ionization because the fragmentation is very low. In this kind of ionization we have firstly the ionization of the gases and we obtain the radical cation of methane molecule, it's the first one because it's the most concentrated.

After several steps the charge pass from gases inside the ionization channel to the molecule as protonation, so we have the addition of hydrogen ion.

Atmospheric pressure Ionization (API)

- In atmospheric pressure ionization sources (API) the ions are first formed at atmospheric pressure and then transferred into the vacuum.
- Some API sources are capable of ionizing **neutral molecules** in solution or in the gas phase prior to ion transfer to the mass spectrometer.
- Because after ionization (occurring in liquid or gas phase), no liquid is introduced into the mass spectrometer these sources are particularly attractive for *the coupling of liquid chromatography with mass spectrometry*.

• Electrospray



The electrospray is the most used ionization source in analytical chemistry, in this kind of ionization, the molecules are hydrolysed when they are still in solutions(liquid), in solutions means that they are dissolved in a solvent. Generally, there is a voltage that is +/- 5 kV, so we have the ionization of our molecule already in the capillary, then the formation of a spray and in the end the nebulization of our sample, still liquid.

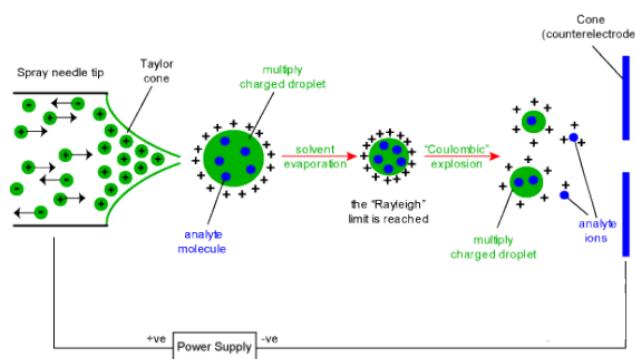
In the left of the

second imagine we can see the capillary and there is the formation of different molecules that are negatively and positively charged. We will analyse separately negative and positive ions. The ionization depend on the molecule structure, can be easily protonated or not.

If we have carbonylic group, we will have negative charged because we have the lost of hydrogen ion for the carboxylic group.

What happens at atmosphere pressure? There is the creation of smallest droplets and then the evaporation of the solvent. The last step is the formation of the single molecule protonated.

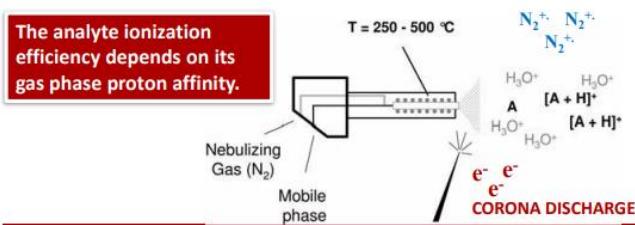
• Electrospray



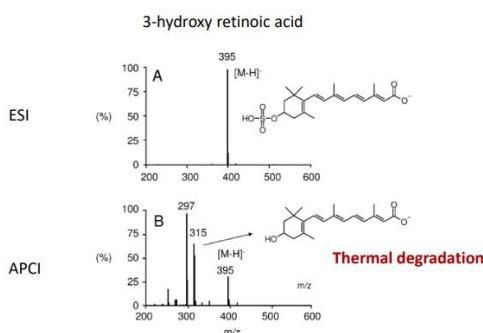
Atmospheric Pressure Chemical Ionization (APCI)

The ionization is at atmospheric pressure but it's occurs when the molecules are already in gas form and it's called chemical ionization because here we have gases that are first hydrolysed and then the charge is transfer to the solid form and to our molecule

- First, an aerosol is formed with the help of a pneumatic nebulizer using nitrogen.
- The aerosol is directly formed in a heated quartz or ceramic tube (typical temperatures 200–500 °C) where the mobile phase and the analytes are evaporated.
- The temperature of the nebulized mobile phase itself remains in the range 120–150 °C due to evaporation enthalpy.
- In a second step, the evaporated liquid is bombarded with electrons formed by corona discharge.



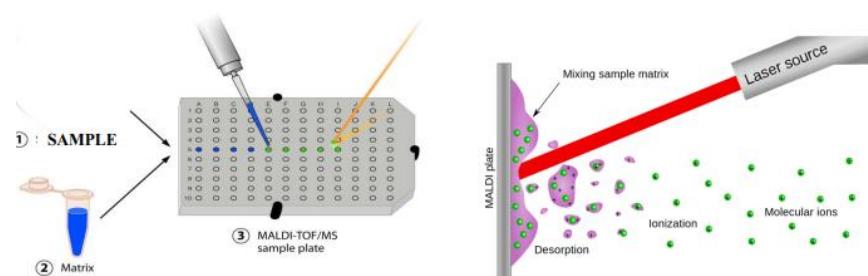
- In positive mode primary ions such as N_2^+ are formed by electron impact.
- These ions react further with water in several steps by charge transfer to form H_3O^+ .
- Ionization of the analyte A occurs then by proton transfer.



Here we have a typical mass spectrometry obtain with electrospray as ionization source. This is a negatively charged, we have a carboxylic acid and a deprotonation.

No fragmentation.

Matrix Assisted Laser Desorption Ionization (MALDI)



MALDI has grown from the efforts to analyze macromolecules by mass spectrometry.

1. A few microliters of solution are spotted onto a MALDI target where the sample crystallizes.
2. After introduction of the target into the vacuum, an UV laser pulse is used to desorb and ionize the sample.

3. Matrix molecules absorb the laser energy and parts of the crystalline surface evaporate.
4. Nitrogen laser emitting at 337 nm is the most widely used.

Depending on the **analytes** that have to be investigated and depending on the provided **laser wavelength**, the appropriate matrix has to be selected.

This technique is more used for proteins and polymers. In this case mass spectrometry is not combined to any separated techniques. In the mass we have the signal of analyte at the same time.

MALDI

- In most cases singly charged ions are detected while very little fragmentation or multiply charged ions are observed.
- MALDI is commonly used for the analysis of high molecular weight compounds such as peptides and proteins, synthetic polymers, DNA and lipids.
- MALDI has the advantage over ESI-LC-MS in that it can achieve a high sample throughput.
- Sample preparation and separation can also be decoupled from the mass spectrometric analysis.

Sinapinic acid Proteins, imaging	α -Cyano-4-hydroxycinnamic acid Peptides, small molecules	2,5-Dihydroxybenzoic acid Proteins
<chem>O=C(Oc1ccc(O)c(C=CC(=O)O)c1)c2ccccc2</chem>	<chem>O=C(Oc1ccc(C#N)cc1)c2ccccc2</chem>	<chem>O=C(Oc1ccc(O)cc1)c2ccccc2</chem>

MASS ANALYZER

OPERATION MODE	Type of Mass Spectrometer
<i>Continuous mode</i>	magnetic sector, quadrupole
<i>Pulsed mode</i>	time of flight
<i>Ion trapping mode</i>	3D or linear ion traps, Fourier transform ion cyclotron, orbitrap

Mass range: The mass range is the m/z range where ions can be detected.

Resolving power (R): The R is the ability of a mass analyser to separate ions of close m/z with similar intensities.

After the evaporation molecules can go inside analyser, that it's able to separate molecules depending of their mass charge ratio.

MASS ANALYSER

Quadrupole-basic analyser

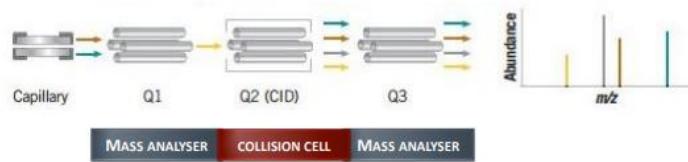
A quadrupole mass analyser is made of four hyperbolic or circular rods placed in parallel with identical diagonal distances from each other. The rods are electrically connected in diagonal. In addition to an alternating radiofrequency (RF) potential (V), a positive direct current (DC) potential (U) is applied on one pair of rods while a negative potential is applied to the other pair.

- The mass range of quadrupoles is typically between m/z 5 and m/z 4000.

Triple-Quadrupole -combination of analyser

We don't have fragmentation but this process is useful to identify molecules, we can have many molecules in a very complicated solutions we can have even isobate molecules (same molecular weight).

A triple quadrupole instrument (QqQ) is a combination of two mass quadrupole mass filters (tandem mass spectrometry) separated by a collision cell which is also a quadrupole operating in RF-only mode

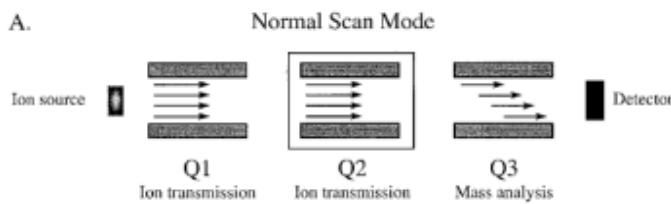


ACQUISITION MODES

Total Ion Current (TIC), or Normal Scan mode

The most basic operation of a mass analyser is to record a “normal” mass spectrum, that is to say a scan through a given appropriate range of masses.

We can have different kind of analyses, different scan mode or acquisition mode.

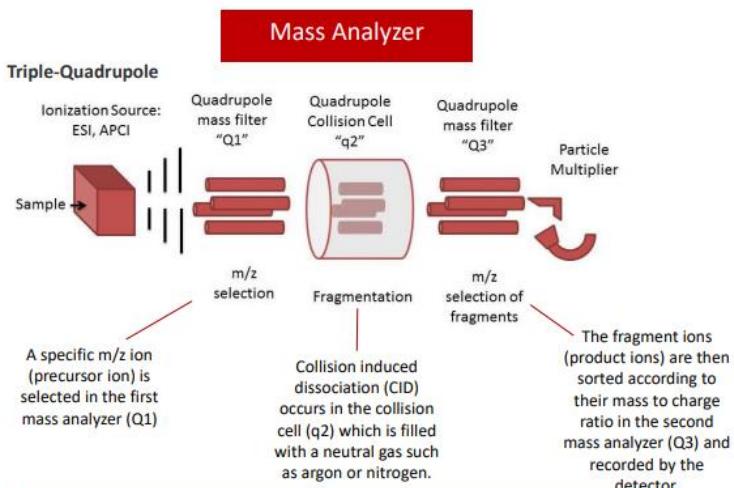


All the ions pass, and they are separated depending on their range of masses.

Q2: switch off

All the ions are detected.

Single or Selected Ion Monitoring (SIM)



Only a limited m/z range is transmitted/detected by the instrument. In this case in the single ion only one ions pass in the system.

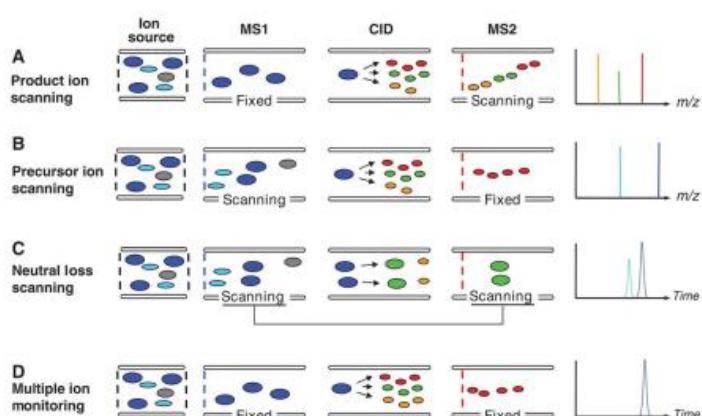
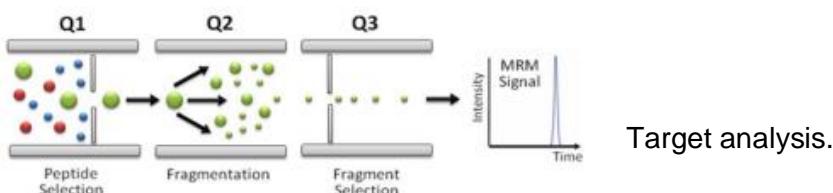
We don't have fragmentation, then we will see that the two most use fragmentation acquisition mode.

Tandem mass, MS/MS, or MS^2

Ions are separated by mass-to-charge ratio in the first stage of mass spectrometry (MS1). Ions of a particular mass-to-charge ratio (precursor ion) are selected and fragment ions (product ions) are created by collision-induced dissociation. The resulting ions are then separated and detected in a second stage of mass spectrometry (MS2).

Selected (SRM) or Multiple Reaction Monitoring (MRM)- using in target metabolomics

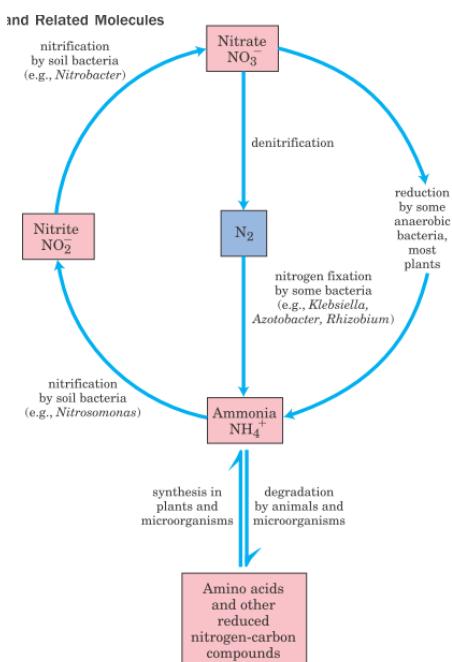
It is method used in tandem mass spectrometry, in which an ion of a particular mass is selected in the first stage (Q1) of a tandem mass spectrometer and an ion product of a fragmentation reaction of the precursor ion is selected in the third mass spectrometer (Q3) stage for detection



Acquisition models

19-Nitrogen metabolism amino acids and nucleotides

NITROGEN METABOLISM



The nitrogen metabolism includes the biosynthesis of amino acids and nucleotides and usually these two metabolisms are explained together because they are strictly interconnected and quite complicated (we'll see the reactions but I don't ask you to remember them all.)

For cells nitrogen is not easily available. In fact only some bacteria, such as cyanobacteria, are able to fix nitrogen from the atmosphere, all the other organisms depend on this metabolism for the production of ammonia from these types of bacteria. Plants, and other bacteria, are able to absorb ammonia into amino acids, in this way nitrogen can enter inside to the other metabolisms and, on the contrary, animals cannot absorb direct ammonia but can intake ammonia from diet and after the nitrogen is used for all the metabolisms.

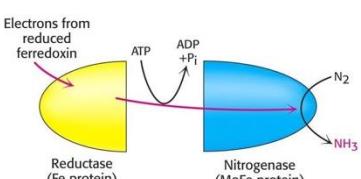
In general the ammonia metabolism is very regulated because cells try to absorb ammonia but they cannot accumulate it. So, as we have seen in the urea cycle, when ammonia is too much, it is released in the environment. If it's possible animals in particular try to savage nucleotides and amino acids to avoid the dependence on diet for ammonia.

To fix some concepts:

Nitrogen is only fixed by some bacteria to form ammonia and ammonia is intaken in plants because they are able to absorb ammonia. Nitrogen, after the degradation by living cells, is released in the environment and ammonia is oxidized into nitrites (NO_2^-) and nitrates (NO_3^-). They also can be absorbed by plants. Part of the nitrates are again oxidized and degraded to form nitrogen. This is the nitrogen cycle for the organism.

NITROGEN FIXATION

Only certain prokaryotes can fix atmospheric nitrogen: These include the cyanobacteria of soils and fresh and salt waters, other kinds of free-living soil bacteria such as Azotobacter species, and the nitrogen-fixing bacteria that live as symbionts in the root nodules of leguminous plants. The first important product of nitrogen fixation is ammonia. This reaction requires a lot of ATP. In fact nitrogen is very stable so it requires a lot of energy to overcome the activation energy for nitrogen.

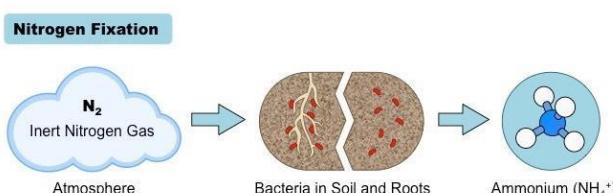


The enzyme that catalyzes this reaction is the nitrogenase complex: this is formed by two domains with two different enzymatic activities: Reductase, which provides electrons, and Nitrogenase, which uses these electrons to reduce N_2 to NH_3 . These proteins work as a dimer and in these two domains we have part of the enzyme in which we have electron carriers that are formed by iron sulfur proteins and these electron carriers can take electrons from ferredoxin. Ferredoxin can be one of the products of photosynthesis, if you remember the electron from photosynthesis can reduce ferredoxin or the protein that can be used for the reduction of NADP^+ into NADPH .

So ferredoxin can give electrons to the nitrogenase complex and electrons are transported by the iron sulfur proteins inside to the first domain of the enzyme.

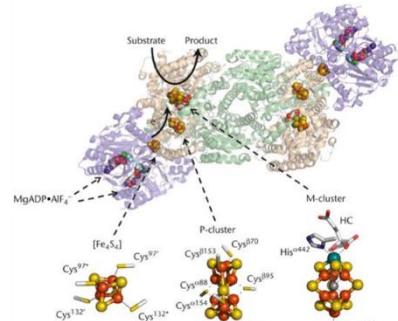
After the hydrolysis of ATP, the enzyme undergoes a conformational change that allows it to overcome the energy of activation for the reduction of nitrogen to ammonia. In this case the electrons are transferred from iron sulfur proteins to another part of the protein in which we have, as electrons carriers, a protein that is coordinated with a mole of ion and an iron ion, so this is a very complicated molecule.

The stoichiometry of the overall reaction for the fixation of Nitrogen requires 16 ATP. This reaction is very endergonic and requires a lot of ATP to form two molecules of ammonia, ferredoxin can give two electrons each



time and 4 molecules of ferredoxin can give electrons to this reaction. The overall reaction can be written $N_2 + 10H^+ + 8e^- + 16 ATP \rightarrow 2NH_4^+ + 16 ADP + 16 Pi + H_2$

This is the structure of the enzyme, we have an homodimer, two copies of proteins and the domain in which ATP is linked. The hydrolysis of ATP gives the conformational change for the other reactions in which we have the electron transfer coenzymes, iron sulfur proteins, iron and the proteins in which the electrons can be transferred to the Nitrogen to form ammonia. This enzyme is very sensitive to oxygen so it is inactivated in presence of oxygen in 30 seconds (so the half life of these enzymes is 30 seconds in presence of oxygen) so the fixation of nitrogen occurs in anaerobic conditions and, depending on the bacteria that catalyze this reaction, some bacteria are strictly anaerobic; other bacteria like cyanobacteria can compartmentalize this enzyme in an anaerobic part of the cell and in another part of the cell they are able to perform photosynthesis and maintain completely separate the oxygen that is produced in photosynthesis from the nitrogen during the nitrogen fixation. These bacteria are very useful for all living organisms because in this way nitrogen can enter inside to the different metabolic pathways for all organisms. When the nitrogen is reduced to ammonia, plants or other microorganisms are able to absorb it and in this way ammonia can enter inside the nitrogen metabolism in our cells.



NITROGEN ASSIMILATION

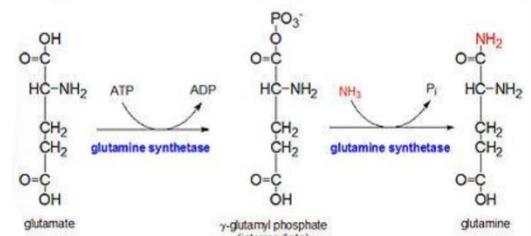
Reduced nitrogen in the form of NH_4^+ is assimilated into amino acids and then into other nitrogen-containing biomolecules. Two amino acids, glutamate and glutamine, provide the critical entry point. The amide nitrogen of glutamine is a source of amino groups in a wide range of biosynthetic processes. Glutamate is the source of amino groups for most other amino acids, through transamination reactions.

The most important amino acid that is involved in the absorption of ammonia is glutamine. From glutamate, through the activity of glutamine synthase, cells can synthesize glutamine, so they can add additional ammonia to glutamate and in this case glutamine is the first amino acid in which ammonia is acquired.

After glutamine and glutamate are used by transaminases, by transamination reaction, to give ammonia to the other reactions for the synthesis of amino acids. This is a reaction catalyzed by the glutamine synthetase, this reaction occurs in two steps. In the first step we have the formation of an intermediate, a phosphorylated form of glutamate, in fact a molecule of ATP is hydrolyzed. After, this is an activated molecule that can react with ammonia to form glutamine.

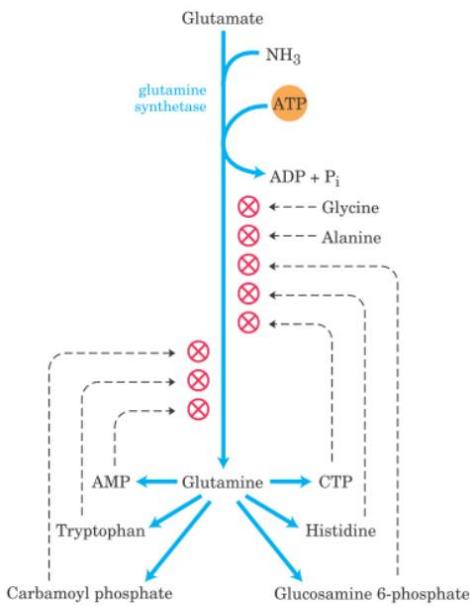
So the entrance of ammonia into the nitrogen metabolism is the formation of glutamine and the enzyme glutamine synthetase is strictly regulated.

In plants this enzyme is able to acquire ammonia from the environment but also in our cells there is glutamine synthase.



REGULATION OF GLUTAMINE SYNTHETASE

Glutamine synthetase is strictly regulated and this regulation is one of the most important things that we have to remember in this metabolism. Glutamine synthetase is regulated at allosteric level but also it is regulated by a covalent modification. Glutamine synthetase is inhibited by a lot of molecules that essentially are the products of nitrogen metabolism. For example you can see that AMP is an inhibitor because the synthesis of purines and pyrimidines are connected to the presence of glutamine. So when we have a lot of nucleotides they are inhibitors of glutamine synthetase but also a lot of amino acids are inhibitors for glutamine synthetase: Tryptophan,



Carbamoyl phosphate, that is an intermediate for the synthesis of pyrimidines, Glucosamine 6-phosphate, that is one the most important sugars, Histidine, glycine and Alanine.

All these inhibitors are able to interact with an allosteric site and they can partially inhibit glutamine synthetase. In this way, depending on the concentration of these different regulators, the activity of glutamine synthetase can slow down step by step. So, for example, if we have only a high concentration of Glycine the activity of glutamine synthetase is not completely inhibited but is only slowed down; in this way part of the glutamine is synthesized for the other molecules but, if you have an accumulation of Glycine, alanine and so on, the activity of glutamine slows down step by step, depending on the concentration of the different inhibitors.

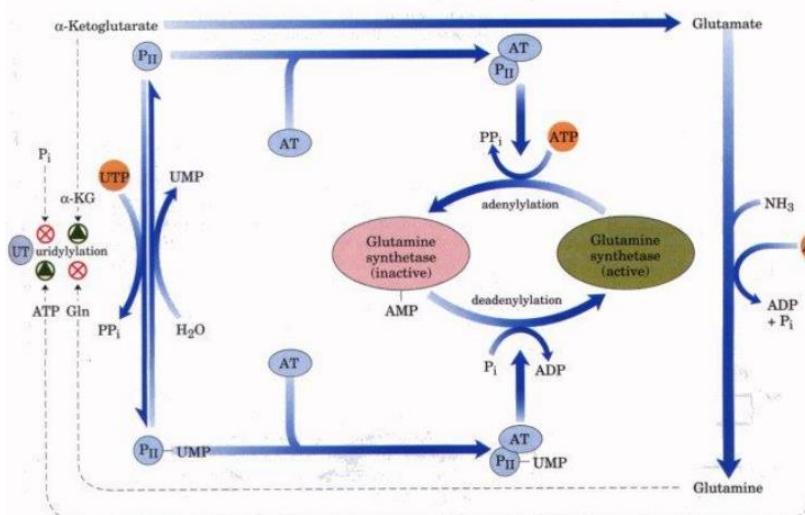
In this way a little bit of glutamine is virtually synthesized and a basic production of some of these reagents, in particular nucleotides, is maintained and it is completely inhibited only when cells are very full for all these intermediates.

This is the scheme for the regulation of glutamine synthetase after covalent modification. Glutamine synthetase can be adenylated, so a molecule of AMP can be added to the glutamine synthetase and it becomes inhibited. On the contrary, when glutamine synthetase is not linked with AMP, it is active.

The enzyme that can adenylate or deadenylate the glutamine synthetase is called adenylyltransferase (AT). This enzyme is able to transfer the AMP group to the glutamine synthetase, hydrolyzing a molecule of ATP; the same enzyme is also able to deadenylate glutamine synthetase, and in this case glutamine synthetase is active. This adenylyltransferase is regulated by another protein, that is called PII(P two). When PII interacts with adenylyltransferase the enzyme is active and it's in its adenylating form so it can react with ATP and can adenylate glutamine synthetase. When the regulator protein PII is uridylated, so in this case we have the addition of another molecule of UMP, the regulator protein activate the adenylyltransferase in its activity that is the activity of adenylylation. So when PII links to AMP, the AT can break the link of AMP to the glutamine synthetase and glutamine synthetase is active.

On the contrary, when the protein pII is not uridylated, AT can catalyze the reaction of adenylylation of glutamine synthetase, inactivating it.

How is regulated protein PII? It is regulated by an enzyme that is called UTP, this enzyme can react with the molecule of UTP to form the PII uridylylate. This enzyme, that is an enzyme that can uridylate protein PII (there is also another enzyme that can deuridylate it), can break the link between UMP to PII, PII is free from the link to UMP and this case PII can activate the adenylylation activity of AT. In this case glutamine synthase is inhibited.



What are the molecules that can regulate the activity of the uridylation enzyme? The activators are: the alpha-Ketoglutarate and ATP. They are essentially the two substrates that are important for the synthesis of glutamine, because alpha-Ketoglutarate is precursor of glutamate, that is the substrate of glutamine synthetase, and the ATP is one of the substrate for the synthesis of glutamine, together with ammonia.

So when alpha-Ketoglutarate and ATP are present in high concentration in our cells, this means that cells have energy to produce glutamine and alpha-Ketoglutarate and ATP can activate the uridylylation enzyme, and this enzyme can uridylate PII, PII is uridylated can react with the AT, and the AT now has the activity of deadenylation and glutamine synthetase is active.

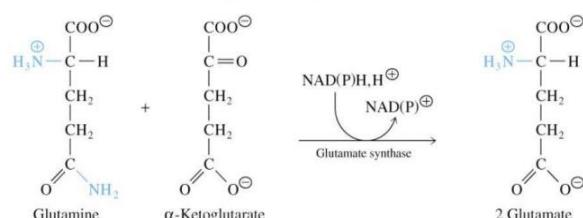
On the contrary, when we have an excess of glutamine and inorganic phosphate, so this means that we don't have a lot of energy and we have a lot of glutamine, in this case the synthesis of glutamine is inhibited, and in fact the uridylylation enzyme is inhibited so we have more PII free, not uridylated, so PII can interact with AT, and AT in this way has the activity of adenyllylation and glutamine synthetase is inhibited.

The synthesis of glutamine is strictly regulated because we have an allosteric regulation and a non-covalent regulation of glutamine synthetase that is regulated by this specific path.

[in the exam: she gives us the scheme, in which she deletes some words.]

NITROGEN ASSIMILATION

One of the first reactions in which nitrogen is transported inside to the cell is the amination of alpha-Ketoglutarate and, in fact, in bacteria and plants these reactions are very active and glutamine can donate an ammonia group to alpha-Ketoglutarate to synthesize two molecules of glutamate.



We can use glutamate by, for example, transaminases for the synthesis of the different ATPs.

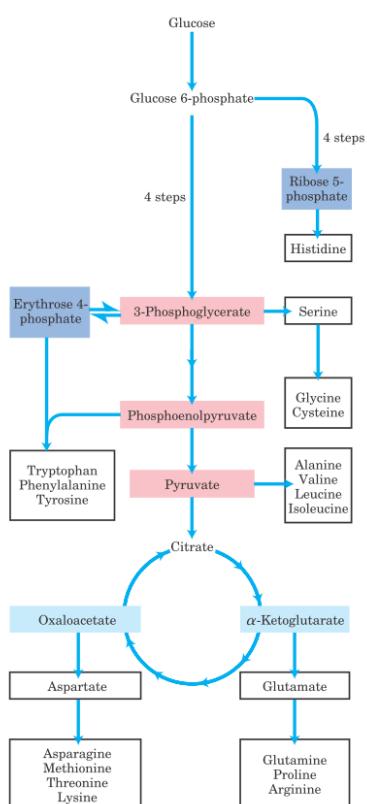
In this reaction we have a reduction and usually NADH, or NADPH, is present as an electron donor.

BIOSYNTHESIS OF AMINO ACIDS

All amino acids are derived from intermediates in glycolysis, the citric acid cycle, or the pentose phosphate pathway. Nitrogen enters these pathways by way of glutamate and glutamine. Ten of the amino acids are just one or several steps removed from the common metabolite from which they are derived. The biosynthetic pathways for others, such as the aromatic amino acids, are more complex. A useful way to organize these biosynthetic pathways is to group them into six families corresponding to their metabolic precursors

More or less similar to the degradation, you remember that amino acids have a carbon skeleton, deriving from alpha-Keto Acids, and during the degradation we have the aminoacid, that is deaminated and the carbon skeleton can be modified to enter in the metabolism of carbon, like the glycolysis, gluconeogenesis, krebs' cycle etc.. For the biosynthesis is the same but in the reverse way.

For the synthesis of the amino acids, the carbon skeletons, deriving from pentaphosphate pathway, glycolysis, gluconeogenesis, and Krebs' cycle, are rearranged to form the carbon skeleton of the different amino acids, and in one of the last steps of the synthesis we have the transaminase reaction, the reverse reaction that we have seen in degradation of amino acid. The same enzyme catalyze this reaction, depending on when the degradation of amino acid is activated, in this case we have the deamination of amino acids, on the contrary when in cells the synthesis of amino acids is activated we have the opposite reaction catalyzed by the same enzyme because they are reversible reactions.



So the most important thing that we have to remember is that, depending on the different aminoacids, we start from different carbon skeleton; for example Histidines derives from the modification of the ribose pyrophosphate and so it is strictly dependent on pentaphosphate pathway; serine, for example, derives directly from 3-phosphoglycerate and from serine cells can synthesize glycine and Cysteine.

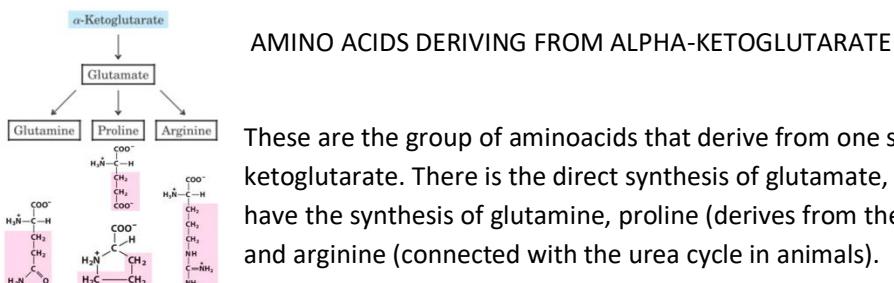
The aromatic amino acids are synthesized from erythrose 4-phosphate that derives from the pentaphosphate pathway with the addition of phosphoenolpyruvate that is an intermediate of glycolysis or gluconeogenesis.

Alanine, Valine, leucine and isoleucine derive from pyruvate, and you see that from Oxaloacetate in Krebs cycle, we can synthetize directly aspartate because Oxaloacetate is the carbon skeleton of aspartate, with transamination reaction we can synthetize aspartate and from aspartate several aminoacids can derive like Asparagine, Methionine, Threonine e Lysine. Alpha-Ketoglutarate is the carbon skeleton of glutamate, and from glutamate cells can obtain Glutamine, Proline and Arginine.

Again the synthesis of amino acids is very complicated and can be different in different organisms, so we don't study, step by step all these reactions for the synthesis of 20 amino acids but it is important that you remember the group of aminoacids that derive from a carbon skeleton and another carbon skeleton (e.g. Aspartate derives from Oxaloacetate, glutamate derives from Alpha-Ketoglutarate, Alanine derives from the direct amination of Pyruvate).

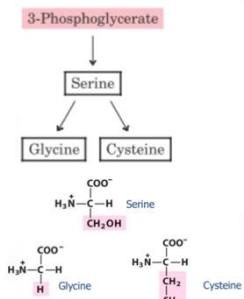
ESSENTIAL AMINO ACIDS

Plants, bacteria, fungarian etc. are able to synthesize all the 20 types of amino acids and, on the contrary, mammals are not able because they have a very rich diet and, in this way, they can intake amino acids and they lost the ability to synthesize all the aminoacids. We have several aminoacids that are called essential amino acids that cannot be synthesized by our cells and we have to take these aminoacids in our diet. They are: Histidine, Isoleucine, Leucine, Lysine, Methionine (Cysteine depends on Met), Phenylalanine (Tyrosine depends on Phe), Threonine, Tryptophan , Valine

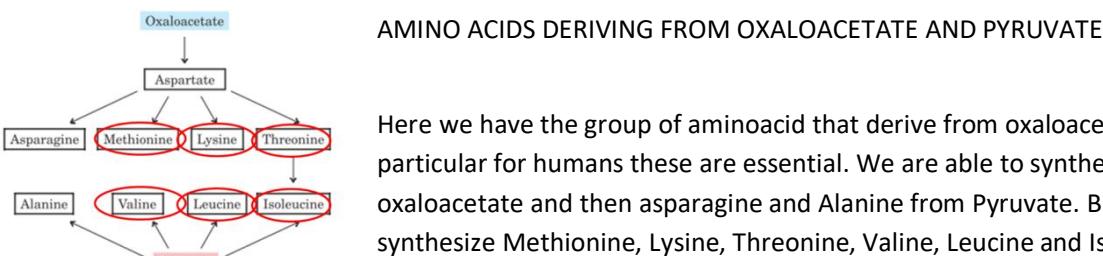


These are the group of aminoacids that derive from one specific carbon skeleton: alpha ketoglutarate. There is the direct synthesis of glutamate, from amination, and from glutamate we have the synthesis of glutamine, proline (derives from the cyclization of the chain of glutamine) and arginine (connected with the urea cycle in animals).

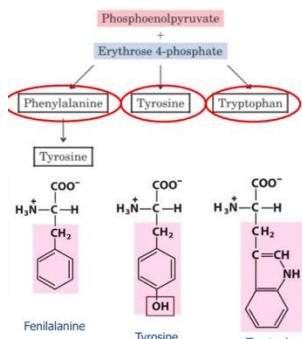
AMINO ACIDS DERIVING FROM 3-PHOSPHOGLYCERATE



This is the group of aminoacid that derive from 3-Phosphoglycerate, so from glycolysis or gluconeogenesis. The first one is Serine, and after, from Serine, cells can synthesize Glycine and Cysteine; in Cysteine it's acquired a sulfur atom to form the sulphydryl group, important for a lot of reactions.



Here we have the group of aminoacid that derive from oxaloacetate and pyruvate. In particular for humans these are essential. We are able to synthesize aspartate from oxaloacetate and then asparagine and Alanine from Pyruvate. But we are not able to synthesize Methionine, Lysine, Threonine, Valine, Leucine and Isoleucine; these aminoacids are synthesized by plants, by bacteria and we can intake these aminoacids by diet.

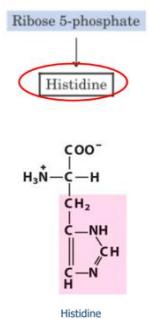


AMINO ACIDS DERIVING FROM PHOSPHOENOLPYRUVATE AND ERYTHROSE PHOSPHATE

Again here we have the precursor for the synthesis of aromatic amino acids like Phenylalanine, Tyrosine, Tryptophan. They derive from a combination of molecules that derive from glycolysis, like phosphoenolpyruvate, and pentaphosphate pathway.

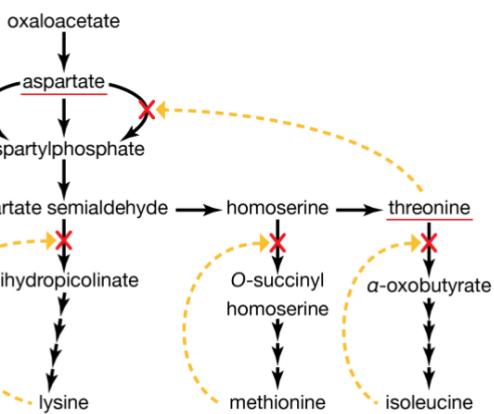
These, for us, are essential amino acids and we cannot synthesize them.

HISTIDINE BIOSYNTHESIS USES PRECURSORS OF PURINE BIOSYNTHESIS Histidine derives from Ribose 5-phosphate and also derives from a little part of the purine ring of ATP. In the synthesis of nucleotides, amino acids are strictly involved, and this is the opposite case in which an amino acid derives from an intermediate of the synthesis of a nucleotide. Indeed, when we have a low amount of amino acids in our cells, one of the first consequences is a reduction in the synthesis of nucleotides and proteins.



REGULATION OF AMINO ACID SYNTHESIS

The regulation of amino acid synthesis depends on the different pathways and different amino acids that cells want to synthesize but, in general, it is regulated in a feedback negative way. In fact, as we have seen from precursors like oxaloacetate or alpha-ketoglutarate or other carbon skeleton precursors, we can synthesize different amino acids.



The pathways for the synthesis of amino acids are branched, for example from oxaloacetate we have seen we have the synthesis of aspartate but also the synthesis of lysine, methionine, isoleucine.

The reactions, sometimes, are branched. There is a sequence of reactions for the synthesis of lysine and the aspartate semialdehyde is a common precursor for lysine but also for the other amino acids. Also homoserine is a common precursor because from it cells can synthesize methionine, threonine and then, from threonine, also isoleucine.

The regulation of this pathway depends on the concentration of the products in the feedback negative mechanism; for example: if lysine is

accumulated, lysine inhibits, in a feedback negative mechanism,

the first enzyme after the aspartate, and in this way lysine inhibits its synthesis but the intermediate that is important for the synthesis of the other amino acids is not inhibited.

If lysine is at high concentration it can also inhibit one of the first reactions of all the pathways after aspartate. But lysine can partially inhibit this reaction, so the synthesis of aspartate is reduced but not completely inhibited and the metabolism is shifted versus the formation of the other amino acids.

The synthesis of aspartate is not inhibited because the aspartate is the precursor of the other amino acids.

When methionine is accumulated it inhibits the first step of the synthesis after the branching, so homoserine is already synthesized and the threonine can be synthesized. Isoleucine inhibits its synthesis after threonine.

Only when threonine is accumulated, and this means that isoleucine, methionine and lysine have inhibited their pathway, in this case threonine can inhibit one of the first reactions.

Only when both lysine and threonine are accumulated, aspartate is not metabolized to form the other amino acid. So the concept is: the product inhibits in a feedback mechanism the synthesis at the level of the branch, in this way the other

Amino acid	Derivatives
Arginine	Creatine
Aspartate	Purines
Glutamate	γ-Aminobutyrate
Glutamine	Purines, pyrimidines
Glycine	Purines, heme, creatine
Histidine	Histamine
Methionine	Spermine, spermidine, S-adenosylmethionine, creatine, choline
Serine	Choline, ethanolamine
Tryptophan	Melatonin, serotonin
Tyrosine	Thyroid hormones, melanin, adrenalin, noradrenalin, dopamine

amino acids can be synthesized and when the amino acids at the end of the metabolism are accumulated, they can inhibit at an higher level all the metabolic paths.

BIOSYNTHESIS OF NUCLEOTIDES

The most important thing that you have to remember about biosynthesis of nucleotides is that it is strictly dependent on the availability of amino acids and it depends on the presence of ribose 5-phosphate that derives from the pentaphosphate pathway.

So in particular for the synthesis of purines, the availability of ribose 5-phosphate is the first step that regulates the synthesis of nucleotides. If we have enough ribose 5-phosphate the biosynthesis of purines slows down.

You know that in nucleotides we have nitrogen bases, so obviously the presence of ammonia, that derives from aminoacids, and CO₂, that derives from the atmosphere, are involved in the synthesis of nucleotides.

Cells are not able to store nucleotides and they continuously synthesize nucleotides, depending on the needs of the cells. For example, the pools of nucleotide are small (ATP is higher because it is important also for the energy) and more or less, in our cells, we have 1% or less of the amount required for the synthesis of DNA or RNA, because they are not stored so our cells continuously synthesize nucleotides for the synthesis of rRNA and the synthesis of DNA during replication.

Because of the importance of this process in dividing cells, agents that inhibit nucleotide synthesis are really important to modern medicine: in particular most anticancer drugs act on the synthesis of nucleotides, because cancer cells are actively dividing and so they act inhibiting the synthesis of nucleotides for replication of DNA.

A nucleotide is formed by: a nitrogen base, sugar (ribose or the deoxyribose) and the phosphate group.

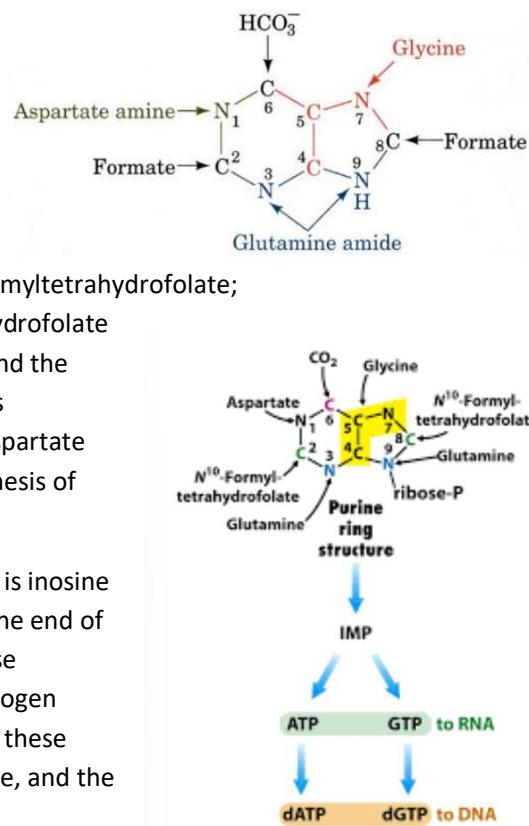
The phosphate group is not present if we are talking about nucleoside, eliminated also the sugar we have the nitrogen bases, that are:

- purines (adenine and Guanine that are formed by two heterocyclic rings because they have nitrogen inside, these two rings are aromatic ring and imidazole ring)
- pyrimidines (an aromatic ring with some substitution in which we have nitrogen; for pyrimidines we have Cytosine, Thymine and Uracil, and Uracil is present only in mRNA)

DE NOVO PURINE NUCLEOTIDE SYNTHESIS

This is a general ring of purine nucleotides and in this slide, the molecules that form the nucleotide purine are highlighted. Glycine gives a lot of atoms for the formation of this ring to carbon and nitrogen; two other nitrogen groups derive from glutamine and the last nitrogen derives from aspartate. So you see that 3 amino acids are involved in the synthesis of purines, one carbon derives from the carbon dioxide(CO₂) and the other two carbons derive from Formate. Formate is a molecule that derives from formyltetrahydrofolate; Tetrahydrofolate is a vitamin that is intaken with diet and, in generale, Tetrahydrofolate can transport one carbon group (for example methyl group or formyl group) and the only group that is not transported by Tetrahydrofolate is carbon dioxide that is transported by another vitamin that is called biotin. Glycine, Glutamine and Aspartate are the three aminoacids that are involved in the synthesis of purines, so synthesis of nucleotides is strictly interconnected with the synthesis of aminoacids.

In general during the synthesis of purine, the first molecule that is synthesized is inosine monophosphate(IMP, not a purine) that is the precursor for ATP and GTP. At the end of the first reaction of purine synthesis the two rings are synthesized on the ribose phosphate molecule that is phosphorylated, so at the end we don't have a nitrogen bases that is linked with ribose and the phosphate but we have the building of these molecules directly on a molecule of ribose phosphorylated to form a nucleotide, and the first nucleotide is inosine monophosphate.



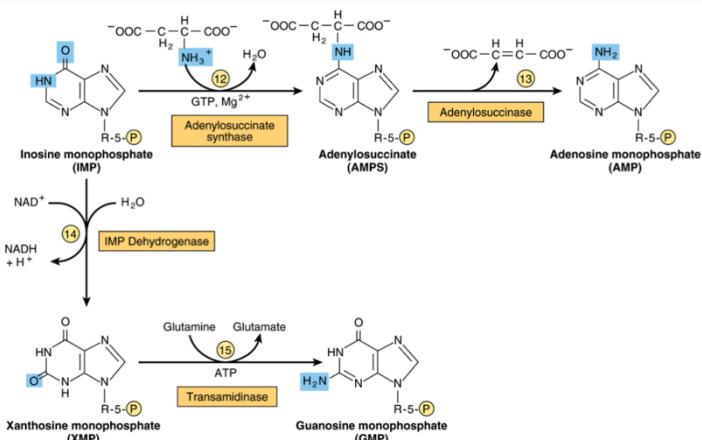
After the inosine is modified to form ATP and GTP and these are the ribonucleotides and again the ribose group is reduced to form the deoxyribonucleotides for the synthesis of DNA. These are the most important steps of the synthesis of purines.

The synthesis of purine starts from the molecule of ribose 5-phosphate that is phosphorylated, so it is activated, to a molecule that is called phosphoribosyl pyrophosphate. Phosphoribosyl pyrophosphate because after the hydrolysis of a molecule of ATP, a pyrophosphate group is added to the first carbon of the ribose.

Directly from the pentaphosphate pathway we have ribose 5-phosphate, now ribose 5-phosphate is phosphorylated to Phosphoribosyl pyrophosphate. This reaction is essentially regulated by ATP because if cells have a lot of ATP they are able to synthesize nucleotides, are able to divide themselves and are able to activate the DNA synthesis.

On the initial ring of Phosphoribosyl pyrophosphate the different substrates react in a several steps reaction, to build the IMP. The amino acids involved in these reactions are: lysine, folate, aspartate, formate.

(we should be able to comment on pictures like this one to give some information about the synthesis of purine)



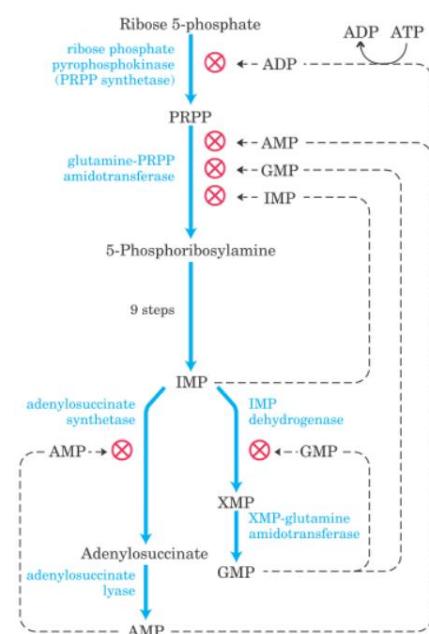
Inosine monophosphate (IMP) can be modified to form adenosine monophosphate or guanosine monophosphate. After the amination of the carbonyl group of inosine you can produce the AMP (Adenosine Monophosphate) and, in this case, the aspartate is used to give the amino group of our molecules. This is important because in this way we have the carbon skeleton of our amino acid that can enter in all the metabolism of carbon and all the amino group is donated for the synthesis of AMP. In this case the aminoacids are essentially used as donors of ammonia, because ammonia is spared in our cells and it's recycled in the different nitrogen metabolism. In this way we produce

AMP but we can also, from inosine monophosphate, synthesize Guanosine monophosphate(GMP). In this case we have first an oxidation to form a carbonyl group and after an amino acid, glutamine in this case, can give ammonia to synthesize GMP. This is the De Novo synthesis of purine. For De Novo synthesis we consider the synthesis starting from precursors that are not nitrogen bases. This path is activated when the cells are not able to recycle nitrogen bases deriving, for example, from diet.

The synthesis of purines is regulated and these are the most important steps for the regulation. The first one is the formation of phosphoribosylpyrophosphate (PRPP) and the regulation of this enzyme derives from the energetic charge of our cells. In fact ATP is an inhibitor of this activity because if we don't have energy it has no sense to synthesize nucleotides.

The second step of our reaction, the addition of the first amino group by glutamine in PRPP, is also strictly regulated. AMP, GMP and IMP are all inhibitors of this step since they are the products of our metabolic process. So only when all these three metabolites are accumulated at the same time, they can completely inhibit this pathway. On the contrary, if there are present only one or two of these metabolites, the synthesis is slowed down but not completely inhibited.

However IMP is accumulated only when we have an accumulation of AMP, because AMP can inhibit its synthesis, and GMP, for the same reason. So, when AMP is

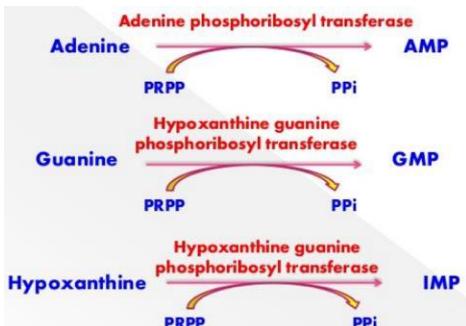


accumulated it can inhibit its synthesis and partially can inhibit the synthesis of nucleotides.

In this way IMP is shifted versus the formation of GMP; this is important because in cells it is required that the synthesis of nucleotides is, more or less, equimolar for the synthesis of DNA or mRNA. However the synthesis of AMP is important also for the synthesis of ATP so the sensitivity to the inhibition of AMP in this pathway is a little bit different.

If GMP is accumulated the synthesis of GMP is inhibited. If both the synthesis of AMP and GMP are inhibited, IMP is accumulated and can inhibit, together with AMP and GMP, the second step of the purine nucleotide synthesis.

PURINE SALVAGE PATHWAY



When our cells have the nitrogen already synthesized, they don't use the De Novo synthesis but they use the purine salvage pathway. In this case the nucleotides are degraded, they are grouped in phosphate, ribose and nitrogen bases, and the nitrogen bases can be used directly for synthesizing new nucleotides adding the nitrogen bases directly with phosphoribosyl pyrophosphate.

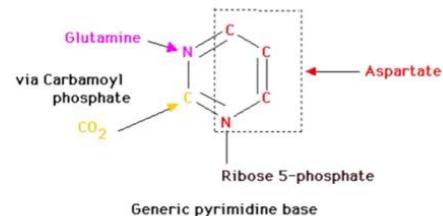
The reaction for the synthesis of AMP is catalyzed by a specific enzyme that is called adenine phosphoribosyl transferase, it transfers adenine to the PRPP and the reaction is an exergonic reaction because here we have the formation of

pyrophosphate. In the case of guanine or hypoxanthine, which is an intermediate for the degradation of purines, the same enzyme is used. Hypoxanthine guanine phosphoribosyl transferase can use both guanine or hypoxanthine, depending on the presence of the nucleotide, to react with PRPP and form respectively GMP and IMP and inosine can be used for the synthesis of GMP and AMP as we have seen before.

De novo pyrimidine nucleotides synthesis.

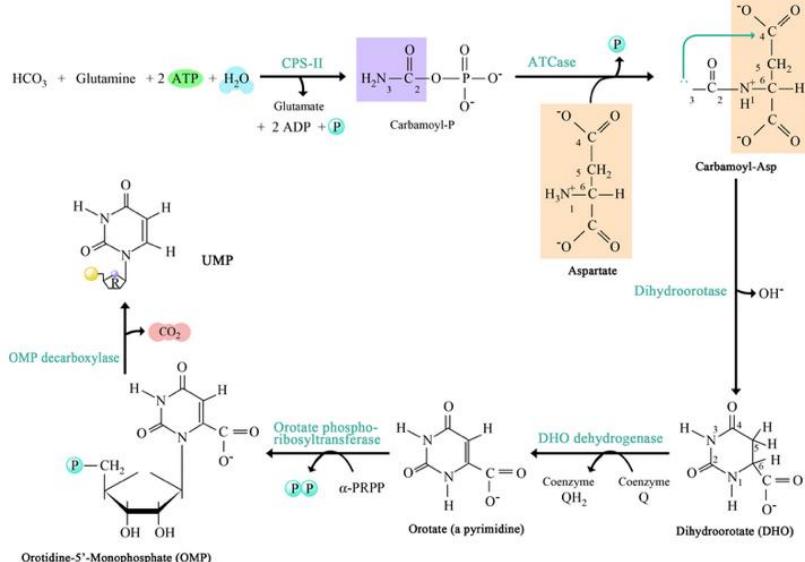
DE NOVO PYRIMIDINES NUCLEOTIDE SYNTHESIS

The precursors for the synthesis of pyrimidines are: aspartate, for nitrogen and a part of the carbon skeleton of the pyrimidine nucleotides, glutamine, a donor of the nitrogen group, and carbon dioxide, donor of the last carbon. Amino acids are involved in the synthesis of nucleotides.



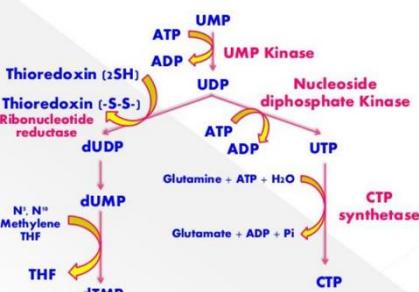
The synthesis of pyrimidines is easier than the synthesis of purines. The first reaction is a reaction that you already saw, since it's similar in the urea cycle. In fact we have the formation of

carbamoyl phosphate that derives from the condensation of an ammonia group, deriving from glutamine, with the carbon dioxide. This is a reaction that requires two molecules of ATPs (also in urea cycle the synthesis is very endergonic and requires ATP). The differences between the reaction seen in urea cycle and here is the donor of ammonia: in urea cycle is the ammonia molecule that derives from the degradation of amino acids, in this case is glutamine. The enzyme that catalyzes this reaction is called carbamoyl phosphate synthetase II to distinguish this enzyme, that is a cytosolic enzyme, from the carbamoyl phosphate synthetase I that is present in mitochondria, in which urea cycle starts. The carbamoyl phosphate



is condensed with a molecule of aspartate to form the first intermediate that is

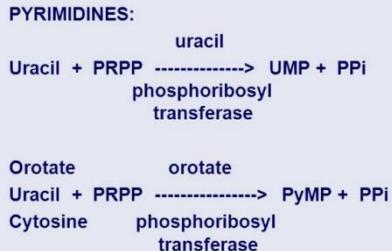
called carbamoyl aspartate, that after undergoes a reaction in which an hydroxyl radical is eliminated and in this way we have the formation of the ring. The enzyme that catalyzes the first reaction of condensation of carbamoyl phosphate and aspartate is called aspartate transcarboxylase. This is a very interesting enzyme because it's strictly regulated, it has an allosteric regulation; it's very important in our metabolism and it's target for several drugs, for the synthesis of pyrimidines etc.. After the formation of the ring we have the formation of the dihydroorotate, this is after oxidized to form Orotate. We have already seen the enzyme that catalyzes this reaction, the DHO dehydrogenase, back in the oxidative phosphorylation. It's important because if we have some problem in oxidative phosphorylation, in particular during the electron transferring from quinol to oxygen, we also have strong problems in the synthesis of pyrimidines and cells cannot survive. Now the orotate can react with the PRPP to form the first nucleotide that is orotate monophosphate (OMP) and after we have the carboxylation to form uridine monophosphate (UMP), this is the first nucleotide synthetized in the de novo nucleotides synthesis.



Now UMP can be modified to synthesize Thymine (TMP) and cytosine (CTP). UMP is usually phosphorylated to form UDP. For the synthesis of CTP we have an extra phosphorylation to form UTP and after the cytosine derives from the deamination of this group of uracil into an amine. The donor of the nitrogen group is glutamine.

For the synthesis of thymine, UDP, a molecule of ribose, is reduced to form deoxyribose (dUDP) and a methyl group is added by the reaction of methyltetrahydrofolate.

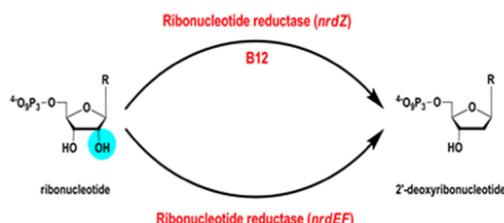
PYRIMIDINES SALVAGE PATHWAY



It is similar to the pathway we saw for purines. Starting from the nitrogen bases, uracil, orotate, cytosine, depending on the availability in cells, these nitrogen bases react with the phosphoribosylpyrophosphate(PRPP) and, thanks to phosphoribosyltransferase, uracil or orotate they form the mononucleotides and after UMP or one of the other nucleotides are phosphorylated as before (in the de novo pathway).

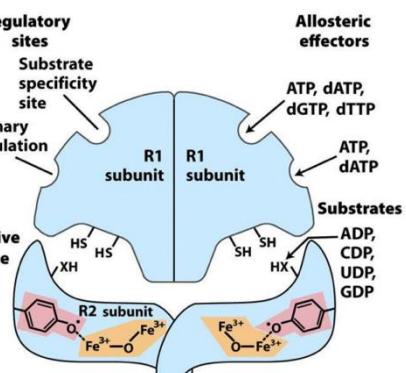
DEOXYRIBONUCLEOTIDES SYNTHESIS AND REGULATION

Deoxyribonucleotides derives from the ribonucleotides, so the synthesis is the same, and after the ribonucleotide, the ribose is reduced to form the 2-deoxyribose to form the deoxyribonucleotides and the reduced position is at the level of the hydroxyl at position two in our sugar. The enzyme that catalyzes (only one enzyme catalyzes this



reaction for all the nucleotides) is the deoxyribonucleotides reductase.

This enzyme has a very complicated reaction because in its catalytic sites, it is able to recognize the different nucleotides depending on the need of the cells, because the synthesis of the deoxyribonucleotides is important that is equimolar, because we need the same concentration of all them. The affinity for the different ribonucleotides is different depending on the necessity of our enzyme. In the catalytic site, the reaction of reduction of this oxydril is complicated, because there is a radical intermediate. The coenzymes that are present in this catalytic site are: iron proteins, in which we have a nucleus



with two iron, vitamin B12 (and in fact, if you don't have enough vitamin B12, the first thing that you can see is anemia) and two cysteines (sulfhydrylic group of cysteine is important to form the intermediate radical for the reduction of the ribonucleotides).

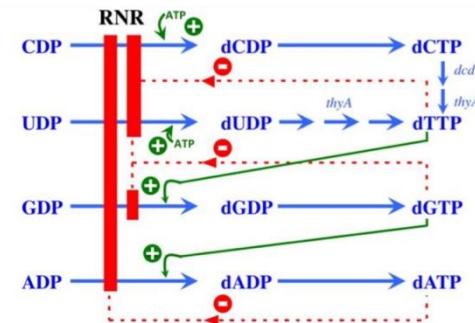
The most interesting thing about this enzyme is that it has two regulatory sites, the first one is the primary regulatory site where we have the general regulation, activation and inhibition of the enzyme.

ATP is a general activator of this activity, in fact when in cells there is a lot of ATP, cells can synthesize the deoxyribonucleotides.

On the contrary, deoxyATP is the general inhibitor, so the accumulation of dATP switches off the activity of this enzyme. But we also have sites of regulation for the substrate specificity, so for regulating the specificity of the catalytic site for the different nucleotides.

And in this way, different nucleotides, like ATP, dATP, dGPT and dTTP are the specific regulators that can modify specificity of the catalytic site for the different nucleotides, and here there is the regulation of this enzyme.

ATP is the activator of our reaction and ATP activates the enzyme, and in this case the catalytic site is more specific for pyrimidines, CDP and UDP. At the beginning, when ATP is present and activates this site, CDP and UDP are reduced to form a dCDP and dUDP. The dCDP and the dUDP after can be modified to form dTTP. When dTTP is accumulated, it can regulate and interact in this site and can modify the affinity for our enzyme for the purine nucleotides, in particular for the GDP. And now the GDP is reduced, and the production of GDP, a part of GDP, is a modulator of the affinity of our enzyme for the ADP. The last product is the dATP. Each nucleotide is a modulator for the affinity of our catalytic site for another nucleotide, before ATP is an activator for the reduction of purines, in general, we have the production of dCTP and the dTTP. The dTTP is a modulator for modifying the affinity of our enzyme for the purines, in particular at the beginning for GDP that is reduced in dGDP and after phosphorylated to dGTP, and dGTP is an activator for the reduction of ATP.



In this way we have the production of the deoxy pyrimidines and after the purines and in this way we have an equimolar production of the 4 deoxyribonucleotides that are important for the synthesis of DNA.

When we have an accumulation of dATP, it is a general inhibitor of the enzyme, and the enzyme is switched off. You can also see some details: the dTTP is an inhibitor of the reduction of UDP, the dGTP is an inhibitor of the reduction of GDP. At the end only ADP can be reduced to dATP.

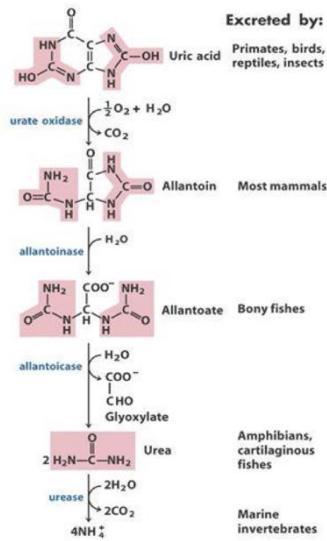
All these regulations occur at the regulatory sites for the specificity of substrate.

PURINE CATABOLISM

At the beginning the cells need to synthesize the new purines and the new nucleotides with purines or pyrimidines. The bases can be used in the salvage pathways, in which the bases are cycled to produce new nucleotides. But we don't have a storage of nucleotides so, if the bases are too much, they are degraded and eliminated. The nucleotides are separated in phosphate ribose and nitrogen bases by several different nucleotidases and at the end we have the formation of the nitrogen bases like inosine, adenosine, hypoxanthine, xanthine and guanine. All these bases are oxidized to form, at the end, Xanthine.

During the degradation of these purines we have the production of nitrogen that is then acquired during the nitrogen metabolism. Another path of nitrogen bases, that derives from xanthine (that is the last nitrogen base after all the amination and oxidation of our nitrogen bases), is further oxidized to form uric acid, and uric acid is a molecule that is enough soluble to be secreted. The last part of the degradation of purines is the reaction

Purine Catabolism

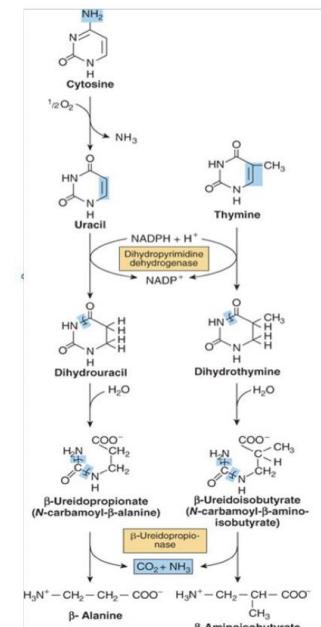


catalyzed by xanthine oxidase. It occurs in peroxisomes, in fact peroxisomes are the organelles in which the molecular oxygen can react to form hydrogen peroxide, and inside to peroxisomes we have the enzyme that it is called catalase that can reduce oxygen peroxide to form water. In primates, uric acid is directly eliminated by kidneys, but uric acid can be modified in other organisms. It can be further oxidized to form Allantoin (most in mammals), Allantoate (another derivative of allantoin), and at the end, in amphibians and cartilaginous fishes is degraded to form urea and ammonia. All these different intermediates are more and more soluble and can be reduced together with water, in particular in fishes.

Pyrimidine catabolism is easier. Cytosine and Thymine are at the beginning reduced and after hydrated to break the ring and to form intermediates that are not directly the amino acids that can be used in protein synthesis but, both beta-Alanine and beta Aminoisobutyrate, are

molecules that can be modified and can be recycled in the carbon skeleton. In this way these molecules are used again or secreted depending on the amount of these molecules.

Pyrimidine Catabolism

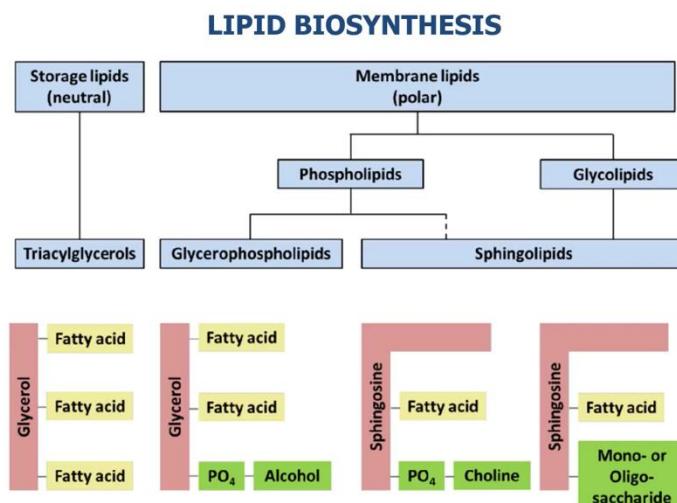


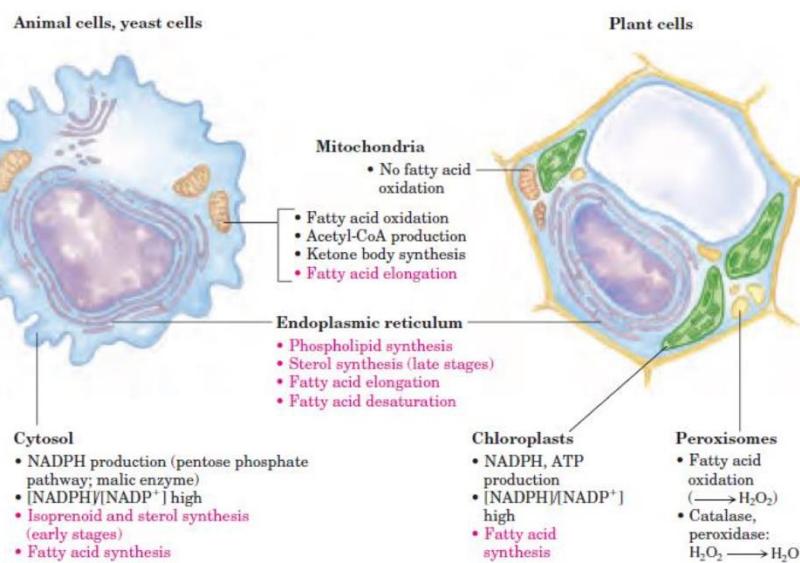
20-fatty acids synthesis

LIPID BIOSYNTHESIS

Lipids are divided into storage lipids (we have Triacylglycerols) or lipids that form the membranes, neutral lipids. Triacylglycerols are formed by a molecule of glycerol that is esterified with 3 fatty acid molecules, these can be saturated fatty acids or unsaturated fatty acids, depending on the fatty acids that our body wants to store. The membrane lipids are polar because they have a hydrophobic part (the part in which we have the fatty acids) and a polar part. The latter in phospholipid is a phosphate group with an alcohol. In other lipids, like glycolipids, is an oligosaccharide group, so one or more molecules of sugar connected together.

However the general organization of membrane lipids is the hydrophobic part, in Sphingolipids, in which we have fatty acids and a long hydrophobic chain that is part of a specific molecule that is called Sphingosine and a polar part. So the membrane lipids are essentially divided into two big categories: phospholipids and glycolipids. The phospholipids are all the lipids in which we have a phosphate group. There are two different types of phospholipids: Glycerophospholipids and Sphingolipids. The Glycerophospholipids have a phosphate group and a molecule of glycerol. The Sphingolipids are molecules with Sphingosine, after we will see what this molecule is. If this molecule is linked with the phosphate, we are talking about phosphosphingolipids and this molecule is in the general group of phospholipids. On the contrary, if we don't have phosphate but only sugars, the name of this phospholipid is glycolipids. [The classification is important, so I hope you will remember this slide!!!!]





The synthesis of lipids, in particular the membrane lipids, occurs in different parts of the cells. The synthesis of fatty acids occurs in cytosol, but after the synthesis of the phospholipids occurs in the endoplasmic reticulum (so the enzymes that can react with glycerol or phosphate in FA ect. are present in the Endoplasmic reticulum).

In the endoplasmic reticulum are present, also, the enzymes that can elongate and unsaturate the fatty acids' chain; in fact we will see that in the cytosol we have the first step for the synthesis of fatty acids, so the enzymes present in the cytosol can synthesize a saturated fatty acids with 16 carbons, the palmitic acids, after the palmitic acids reacts with glycerol or sphingosine and this

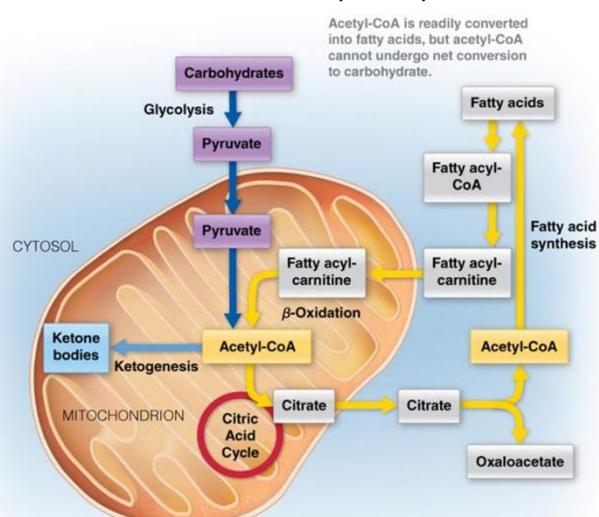
reaction occurs on the surface of endoplasmic reticulum. Cholesterol synthesis occurs in different organelles (a part occurs in Peroxisomes, a part occurs in endoplasmic reticulum), in general we say that the synthesis of cholesterol occurs in the cytosolic part of our cells.

BIOSYNTHESIS OF FATTY ACIDS

The synthesis of fatty acids starts from the molecule of Acetyl-CoA.

Acetyl-CoA is usually produced inside the mitochondrial, it derives from the degradation of CoA, after the decarboxylation of pyruvate. Acetyl-CoA can also derive from the degradation of the skeleton carbon of amino acids. When cells have a lot of nutrients, the nutrients enter in the catabolic pathways, and at the end we have the production of Acetyl-CoA. The energetic fate of Acetyl-CoA is to enter into the Citric Acid Cycle but, if cells have a lot of ATP, the Citric Acid Cycle is inhibited. If you remember, the first reactions of the Citric Acid Cycle are regulated so, in high energetic levels of cells, we have an accumulation of Citrate, the first substrate deriving from the first reaction of Citric Acid Cycle. Citrate, if it is not oxidized, is used for the synthesis of fatty acid, for the storage of fatty acids; from carbohydrates we can synthesize fatty acids, also from aminoacids, but we cannot do the reverse reaction: only plants, some bacteria or some yeast can do this by the glyoxylate cycle.

How does the fatty acid synthesis start?



The first thing is that Citrate has to exit from Mitochondrial, because the synthesis of fatty acid occurs in Cytosol; the beta oxidation occurs in mitochondrial and the synthesis of fatty acid occurs in Cytosol. These two metabolic pathways are one the opposite of the other one, and they occur in different compartments, in this way they are separated and cannot occur at the same time. Citrate exits from the mitochondrial and is splitted in two molecules that are Acetyl-CoA and Oxaloacetate. Oxaloacetate is recycled and it's transported inside to the mitochondrial and Acetyl-CoA is the starting molecule for the synthesis of fatty acid. Several molecules of Acetyl-CoA are condensed to form the first fatty acid, that is the palmitic acid, that has 16 carbons.

CITRATE SHUTTLE

The Citrate shuttle is a Circular mechanism that is used by mitochondria to transport citrate out of mitochondria and to recycle the oxaloacetate and it occurs at the level of the mitochondrial matrix. From the carboxylation of pyruvate, acetylCoA is synthesized and, in the first reaction of Krebs Cycle, we have the formation of citrate.

Now we have an accumulation of citrate, because the krebs cycle is inhibited, so citrate can exit from the mitochondrial through a transporter (in the mitochondrial membrane there is a specific transporter for citrate) and this is a channel that is opened when citrate is accumulated.

After citrate exits from mitochondria, in cytosol there is an enzyme, called citrate lyase, that is able to split citrate in oxaloacetate and Acetyl-CoA. Acetyl-CoA goes to the fatty acid synthesis but now we see the fate of oxaloacetate.

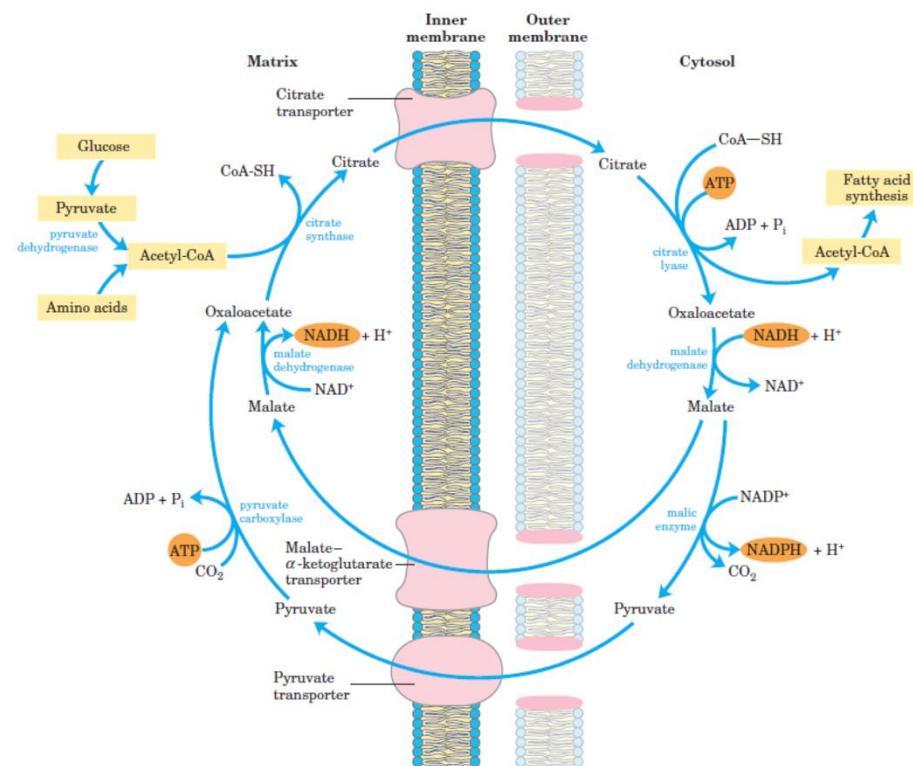
Oxaloacetate in cytosol is reduced to malate, (we have already seen this enzyme, the malate-dehydrogenase, in the malate aspartate shuttle, it is the same), and now malate can directly enter inside the mitochondria through the transporter, the antiporter malate and malate-alpha-

ketoglutarate transporter (that is the same we have seen in malate aspartate shuttle), but malate can also undergo to another reaction in which malate is oxidized and decarboxylated to pyruvate and this by the malic-enzyme. In this case, if the malate is decarboxylated and oxidized to pyruvate, we have the formation of NADPH, because malic-enzyme is an enzyme that has a substrate NADP⁺ that is reduced to NADPH. This reaction is active in particular when the balance between NADPH and NADP⁺ is less than one, so if we have a lot of NADP⁺ and a low amount of NADPH.

Why is the enzyme activated in this case? Because it has the substrate that is activated, and in this way the oxidation of malate allows the production of NADPH that is the reducing power for the synthesis of the fatty acids. The pentaphosphate pathway is active when fatty acid synthesis is active, the oxidative part of pentaphosphate pathway is important when cells are actively dividing, because we have to produce ribose 5 phosphate for the synthesis of nucleotides but also is active when cells want to synthesize fatty acids because in the oxidative part we have the production of NADPH, and NADPH is the molecule that can catalyze the reduction reactions in anabolic pathways, in particularly in fatty acid synthesis.

However if cells need more NADPH, also this reaction, the oxidation of malate to pyruvate, is useful for starting the reaction of fatty acid synthesis, because here we have the synthesis of NADPH. When cells want to synthesize fatty acids, usually they don't need NADH in the matrix because ATP is already present, so often this way is preferred than the direct transfer of malate inside to mitochondria.

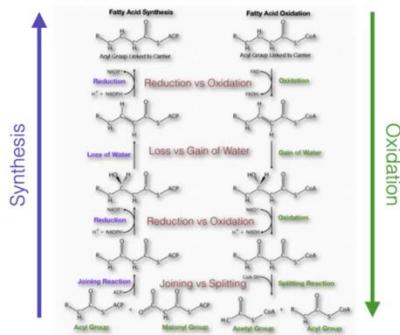
All these reactions are at equilibrium, so the verse of these reactions depends on the requirement of cells. Cells can choose different fates of malate depending on the needs of NADPH essentially, NADPH in cytosol or NADH in malate. Because you see that, if malate directly enters inside to mitochondria, it's oxidized to oxaloacetate by the last reaction of Krebs Cycle producing NADH that is then used in oxidative phosphorylation. However, if malate enters, it is oxidized and it forms oxaloacetate and all the shuttles, depending on the shuttle that cells need, can go on. If pyruvate enters inside the mitochondria it is carboxylated to form oxaloacetate again. Now you have NADPH, because it is produced by pentaphosphate pathway or by this shuttle (in part), and acetylCoA, and these two molecules are the most important molecules for the synthesis of fatty acids.



This is a comparison between the synthesis of fatty acids and the oxidation of fatty acids: four reactions are very similar but they occur in different compartments.

FATTY ACIDS BIOSYNTHESIS

Fatty Acid Synthesis vs Oxidation



During the oxidation we start from an acylCoA molecule that is oxidized, after it is hydrated, then again oxidized and after, at the end, we have the splitting of the betaketoacetyl group into two molecules of acyl group or acetylCoA ,this is the last step, we are starting from the molecule with four carbons. For the synthesis we don't start from two molecules of acetylCoA directly but we start from a molecule of acetylCoA and a malonyl CoA, but malonyl derives from the carboxylation of acetylCoA. MalonylCoA is a molecule that can condense with acetylCOA to form the first molecule similar to the molecule we have seen in beta oxidation because we have the decarboxylation of malonyl (it has three carbons, acetylCoA has two carbons). The carboxylation of the acetylCoA, and after the decarboxylation, gives the energy for the condensation and for producing the first beta keto acid in fatty acids

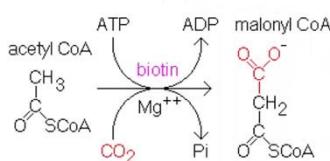
synthesis. Now we have molecules with four carbons and we have a reduction, the opposite of oxidation in this reaction, and NADPH is the donors of electrons, we have the lost of a molecule of water and we have again a second reduction (again the donor of electrons is NADPH), so in the synthesis we need two molecules of NADPH. These are the four central reactions for the synthesis of fatty acids, we have a molecule with four carbons that can react with another molecule of malonylCoA, there is another condensation, so in this case, during all this four cycles of fatty acids, we elongate our chains of a couple of carbons. Essentially it's the same thing in the opposite verve that we saw in fatty acids oxidation.

This is the first reaction for the synthesis of fatty acids in which the acetylCoA is carboxylated to form malonylCoA, this reaction is catalyzed by enzyme AcetylCoA carboxylase. This enzyme contains a coenzyme that is **biotin** (vitamin B1), that we have already seen in pyruvate carboxylase. In general all the carboxylation reactions in which carbon dioxide is involved, are reaction in which the coenzyme is biotin, because biotin is a transporter of carbon dioxide and this reaction requires ATP because is an endergonic reaction, with energy of ATP, the synthesis of AcetylCoA is shifted versus the formation of malonylCoA. This is the first reaction of the fatty acids synthesis and in fact the enzyme of this reaction is strictly regulated. AcetylCoACarboxylase is activated by citrate. From citrate we have the production of AcetylCoA: in the cytosol we have an accumulation of citrate, citrate is splitted into AcetylCoA and oxaloacetate and the citrate is also an allosteric activator of this enzyme. On the contrary an allosteric inhibitor of this enzyme is the product of the synthesis of fatty acid, that is PalmitoylCoA, the last product of the synthesis of fatty acids. This enzyme is also regulated at a covalent level, because it can be phosphorylated, and when it is phosphorylated it is inactive, on the contrary when it is dephosphorylated it is active.

The hormones that can regulate this phosphorylation are all the ones that can activate the protein kinase A, hormones that stimulate the synthesis of cyclic AMP can activate protein kinaseA, and protein kinaseA can phosphorylate this enzyme and inactivate it.

FATTY ACIDS BIOSYNTHESIS

The long carbon chains of fatty acids are assembled in a repeating four-step sequence starting from the molecule of acetyl-CoA that is carboxylate by the enzyme **Acetyl-CoA carboxylase**.



Acetyl CoA is carboxylated to malonyl CoA. This reaction costs the hydrolysis of a molecule of ATP. This energy will be used to drive the condensation of acetyl-CoA with malonyl-CoA to an acyl-CoA.

The first reaction of the fatty acids synthesis pathway is strictly regulated.

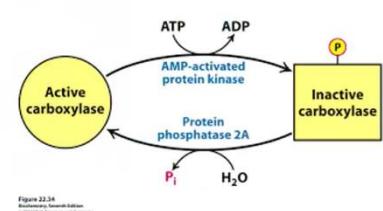
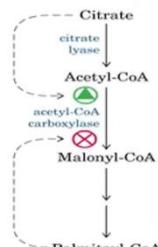
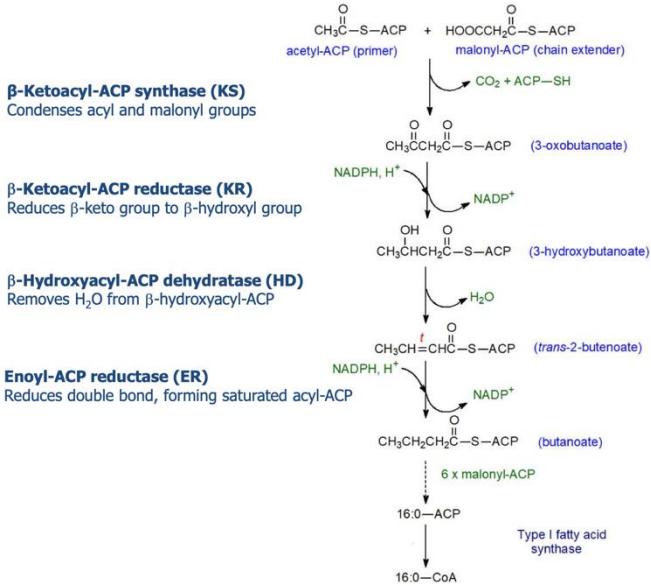


Figure 22.24
Biology, Eighth Edition
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Here we have the four that we have seen before:
The first reaction, where we have synthesized malonylCoA, is not present here and it is the transfer of acetyl CoA and malonylCoA to a protein: ACP. This protein is called A(cyl)C(arrier)P(rotein) and it can transport acyl groups, acetyl or malonyl groups. Essentially it does the same work of coenzyme A, in this case our groups are transported by this protein to the catalytic size of the different reactions for the synthesis of FA. So you see acetylACP, not CoA, because acetylCoA reacts with this protein, that has a sulphydryl group, to form acetylACP, and the same is for malonyl ACP. This reaction is considered only as transfer of acyl groups to the protein but the metabolites are not modified. The first reaction, in which we have another product, is the condensation of acetyl group with malonyl group. Here we have a decarboxylation and it gives the energy for pushing this reaction versus the formation of

beta ketoacyl molecules (we have a keto group in the beta carbon). The enzyme that catalyzes this reaction is called **β -Ketoacyl-ACP synthase (KS)**, also called condensed enzyme, because it catalyzes the condensation reaction. Now this molecule is reduced by NADPH to form the beta hydroxy keto group. The second enzyme that catalyzes the reduction is called **β -Ketoacyl-ACP reductase(KR)**.

The third reaction is catalyzed by an enzyme that can extract a water molecule to form the unsaturated intermediate. It is called **β -Hydroxyacyl** (because this is the substrate in hydroxyacyl) **ACP** (because is linked with the protein) **dehydratase**. From this reaction an enoyl intermediate is formed because we have two double bonds and these double bonds after are reduced by NADPH to form the saturated FA with four carbon. After this molecule can react with another molecule of malonylACP. So at the end we have a condensation, a reduction, a dehydration and a reduction and our molecule is elongated, step by step, by two carbons.

FA Synthase complex

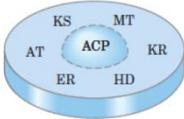
Bacteria, Plants
Seven activities
in seven separate polypeptides



Yeast
Seven activities
in two separate polypeptides



Vertebrates
Seven activities
in one large polypeptide

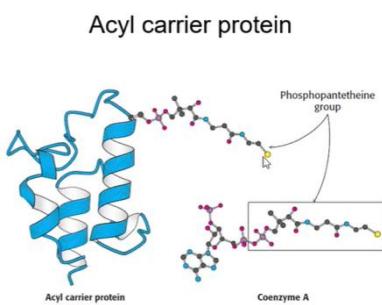


The synthesis of FA is one of the examples of a metabolic pathway because we have the substrate at the beginning and the reaction goes on several times. Only at the end of all the reactions, we have the release of the product, in this case the palmitic acid. The FA intermediates are always attached with some of the proteins of this complex and are not released in the cytosol; we don't have intermediates during the synthesis of palmitic acid. It is a case of a panel(?) pathway in which the intermediates are not interconnected with other metabolic pathways. Only the reagent and the product can be connected with other pathways. In plants and bacteria all the enzymes we have seen, are synthesized in separate polypeptides, and these polypeptides are organized in a super complex in which all these proteins form a quaternary, very big complex, like pyruvate dehydrogenase in which we also have the ACP protein, that is the protein that transport the acyl group, deriving from

elongated FA. During the evolution, the genes for these proteins were fused and in Yeast, for example, we have two polypeptides separated, in which the blue ones (look at the picture) are those that are fused in the same gene. So at the end, during evolution, we have the formation of two polypeptides, and inside these polypeptides we have several enzymatic activities. So we have a polypeptide in which we have an enzymatic activity of the condensation and reduction, the two first steps, and in this protein there is also the ACP protein, and the second polypeptide, a very big polypeptide, in which we have four (grey ones) catalytic sites.

In mammals, in vertebrates, the FA synthase is formed by a single very big protein in which we have several domains, and for each domain we have a catalytic activity. So evolution, considering these panels' metabolic pathway, prefers to produce a very big protein in which, in different parts of this protein, we have different catalytic sites.

The acyl carrier protein

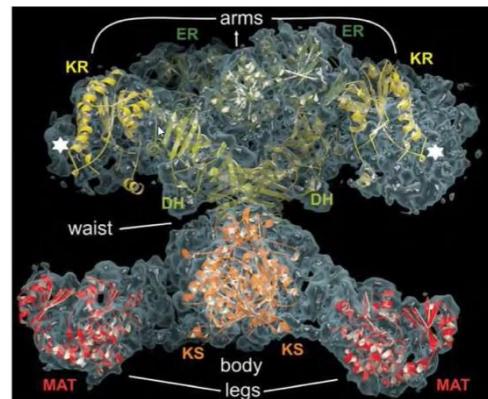


It is a little protein, when it is separated from other proteins, in which we have a lysine group linked with a phosphopantetheine group. This group is the same that is present in CoA. At the end, the active part of CoA is the SH group that can react with the carboxylic part of acetyl, acetate or other carboxylic group, to form, for example, acetylCoA. The group is the same, so the acetyl group or the malonyl group, linked with CoA, are transferred to this group. This protein has a very long arm, it is important because it is the carrier of the different substrates. It can transport these substrates to the catalytic site of different enzymes, regulating the step of the reaction. In the same way, this is similar to pyruvate dehydrogenase.

Mammalian fatty acids synthase

This is the mammals' enzyme for FA synthesis. It works as a dimer, so here we have two copies of these very big proteins and we can see the two polypeptides. The letters indicate the different catalytic parts of this big enzyme complex.

The MAT enzyme is the enzyme that can transfer the malonyl or the acetyl CoA from CoA to the ACP protein; it can transfer only the groups from CoA to the sulphhydryl group of ACP protein. The ACP protein is indicated with a star, in our figure. The long arm can transport substrate in different catalytic sites of the protein, the position of ACP is the part in which acetyl CoA or malonyl CoA can react, and can be transferred to ACP protein.



Malonyl/Acetyl-CoA-ACP transferase (MAT)

Transfers malonyl group from CoA to ACP

Acylic carrier protein (ACP)

Carries acyl groups

β -Ketoacyl-ACP synthase (KS)

Condenses acyl and malonyl groups

β -Ketoacyl-ACP reductase (KR)

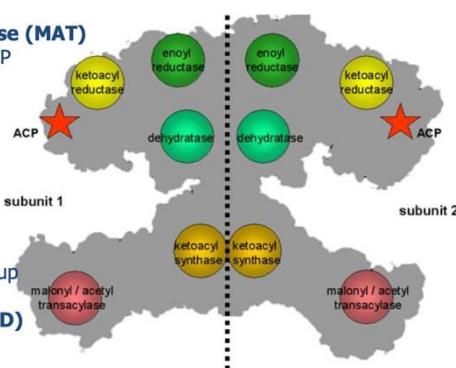
Reduces β -keto group to β -hydroxyl group

β -Hydroxyacyl-ACP dehydratase (HD)

Removes H₂O from β -hydroxyacyl-ACP

Enoyl-ACP reductase (ER)

Reduces double bond, forming saturated acyl-ACP



(KS) Ketoacyl-ACP synthase The KS synthase condenses the enzyme, so the ACP protein transports the substrate here for the condensation and our product is linked with ACP protein. ACP protein can transport the substrate to the other catalytic site(KR).

(KR) beta-ketoacyl-ACP reductase The second one is the reductase site (KR), in which ketoacyl-ACP is reduced. After the reduction, the ACP protein moves its arms to the dehydratase group (HD)

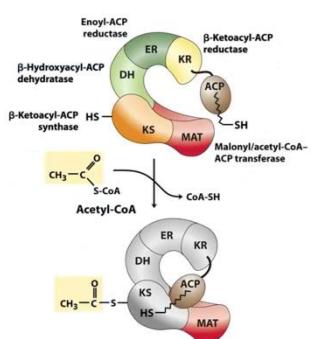
(HD) beta-Hydroxyacyl-ACP dehydratase In the dehydratase catalytic site, we have the loss of a molecule of water and the last reaction, which is the second reduction. After, The ACP protein can transport the little FA to the MAT enzyme in which another molecule of malonylCoA can react with ACP protein and, again, we have the condensation, the reduction, the dehydration and the second reduction. This occurs several times, for 7 times, to form palmitoyl FA or palmitate.

Now we see the different reactions considering one of the two protein (the protein acts as a dimer)

We have the ACP protein with the sulphhydryl group; at the beginning this group reacts with acetylCoA in the MAT enzyme to form Acetyl-ACP-protein.

Immediately the acetyl group is transferred to a sulphhydryl group that we have in the Ketoacyl-ACP-synthase part. The condensing enzyme has,

Reaction 1 – Acetyl-CoA is transferred on Ketoacyl-ACP synthase through the activity of Malonyl/Acetyl-CoA-ACP transferase

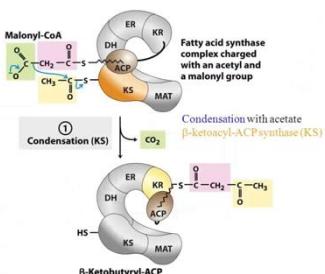


in the catalytic site, a sulphydryl group that immediately reacts with the Acyl Group that is transferred by the ACP protein. In this way, our ACP protein is free to react with the malonylCoA.

Reaction 2 – Malonyl-CoA is transferred on ACP-protein through the activity of Malonyl/Acetyl-CoA-ACP transferase

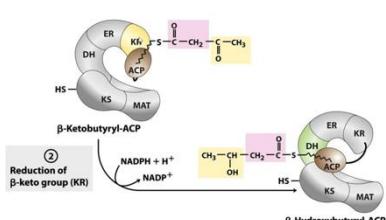
Here we have the MolonylCoA that can react with ACP protein, so we have the malonylACP, and the ACP protein transports the malonyl group to the catalytic site of the condensing enzyme. Here we have the reaction of the condensation of malonylCoA; essentially the acetyl group is transferred to the malonyl group in which we have the decarboxylation with the formation of beta ketoacyl group. The decarboxylation gives the energy for this condensation reaction.

Reaction 3 – Malonyl- and acetyl groups are condensed through the activity of Ketoacyl-ACP synthase



This is the reaction: we have the decarboxylation and, at the level of ACP protein, you see that we have our beta ketoacyl group, and the condensing enzyme, in which we have a sulphydryl group, is freed. The ACP protein transports our new molecule, and this protein can transport this molecule to the second catalytic enzyme that is reducing part, the ketoacyl reductase.

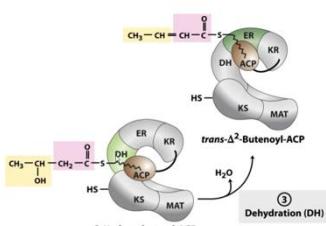
Reaction 4 – Ketobutyryl-ACP is reduced through the activity of Ketoacyl-ACP reductase



We have the reduction, KR. In this catalytic site, a molecule of NADPH can enter and can catalyze the reaction of this carbonyl group (the beta carbonyl group), to form the hydroxyl group.

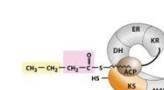
After this reduction, the ACP protein can transport the hydroxylic intermediate to the dehydratase enzyme, in which we have the activity of dehydration.

Reaction 5 – Hydroxybutyryl-ACP is dehydrated through the activity of Hydroxyacyl-ACP dehydratase



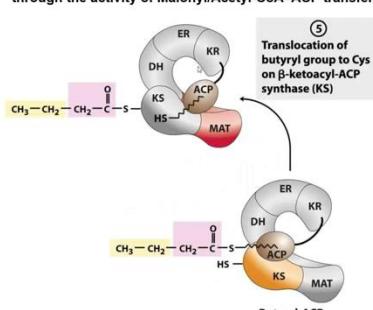
This enzyme can catalyze the dehydration of our Hydroxybutyryl ACP molecule and, after the loss of a molecule of water, we have the formation of our enoyl group with double bond. Now this protein can transport the molecule to the second catalytic site in which we have the reduction, the enoyl reduction.

Reaction 6 – Trans-butenoyl-ACP is reduced through the activity of Enoyl-ACP reductase



In this catalytic site, a molecule of NADPH can give two electrons to saturate our molecule to form the first intermediate, that is Butyryl ACP.

Reaction 7 – Butyryl-ACP is translocated on Ketoacyl-ACP synthase through the activity of Malonyl/Acetyl-CoA-ACP transferase



The last reaction is only a transferring reaction. Our molecules, the ketoButyryl, is transferred from ACP protein to the sulphydryl group of the first enzyme, the ketoacyl synthase enzyme, the condensing enzyme, and in this way the ACP protein is free (because this group is transferred in the catalytic site of the condensing enzyme) and it can react, at the MAT level, with another molecule of malonylCoA to form malonylACP and the reaction can go on for the second round of reactions 7 times.

MAMMALIAN FATTY ACIDS SYNTHASE

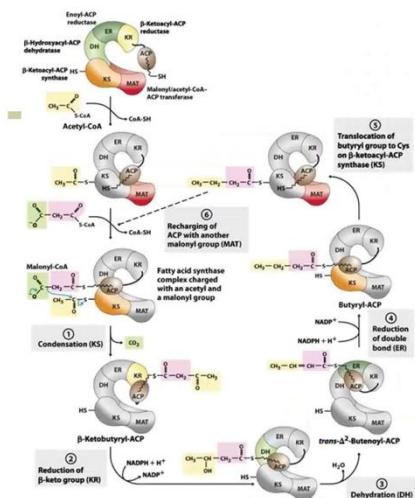
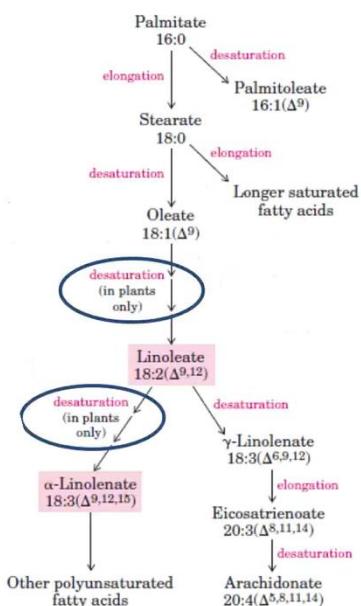


Figure 21-6
Lehninger Principles of Biochemistry, Fifth Edition
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Seven cycles of condensation and reduction produce the 16-carbon saturated palmitoyl group, still bound to ACP. For reasons not well understood, chain elongation by the synthase complex generally stops at this point and free palmitate is released from the ACP by a hydrolytic activity in the complex.

FATTY ACIDS ELONGATION PATHWAYS



The elongation pathway usually occurs in the plasmic reticulum in which we have the enzyme that can elongate, desaturate and modify the palmitate, that is the first FA produced.

Why are the enzymes in the endoplasmic reticulum? Because palmitate now is a Hydrophobic molecule, the enzymes that can catalyze the next reactions are enzymes that are embedded inside to the membrane, in particular in the membrane of endoplasmic reticulum. However, remember that some specific enzymes for the formation of some specific lipids are present also in other membranes like golgi membrane, peroxisome membranes.

All these enzymes are membranes' proteins because substrates are hydrophobic. In our cells, mammals cells, palmitate can be desaturated, so we have the formation of desaturated FA , or it can be elongated to form stearate or oleate and during the different steps, in this case the intermediate are released by the enzyme and they can branched, modified for the production of oleate, or cells can be further elongated.

In mammals cells, not all the FA that are present in our membranes are synthesized in cells, in fact we need to intake some FA by diet, and in particular unsaturated FA that are synthesized only by plants or bacteria called essential FA. Mammal cells are able to modify this essential FA to form other FA like arachidonate. We have to consider that only unsaturated FA, and more complex FA, derive from FA that we have intaken by diet.

After the synthesis of FA we can synthesize lipids.

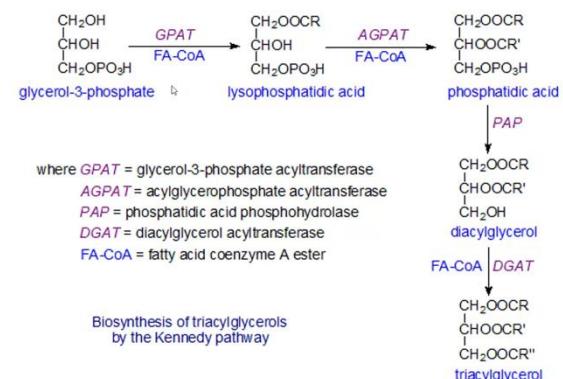
SYNTHESIS OF LIPIDS

Our cells are able to synthesize Triacylglycerols as storage for FA. Triacylglycerols is formed by a molecule of glycerol in which the three hydroxyl groups are esterified with a molecule of FA. The FA can be both unsaturated or saturated, depending on the type of Triacylglycerols .

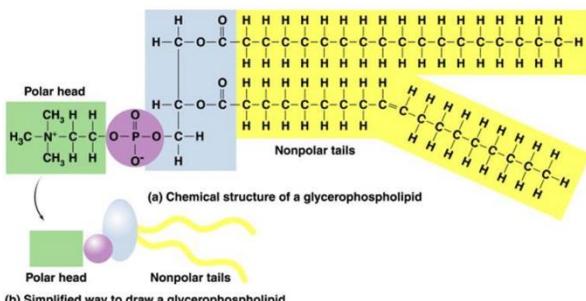
TRIACYLGLYCEROLS

We start with glycerol-3-phosphate that can react with the first FA through an enzyme called GPAT (acyltransferase means that the FA is transferred onto a glycerol-3-phosphate to form this intermediate that is called lysophosphatidic acid). After, a second molecule of FA is added, in the central hydroxyl group, by another acyl transferase (it is important to remember the step, not the name); the step starts with a glycerol phosphate, that is an activated molecule, we have the first FA in the carbon one, then we have the second FA in the carbon two, by the esterification of the second hydroxyl. Usually the first FA is a saturated FA and the second one is an unsaturated FA. The phosphatidic acid is formed. It is the simplex phosphoglycerolipids, and so in some cases it is used for the synthesis of phospholipids. In this case we want to synthesize triacylglycerol so phosphatidic acid is dephosphorylated to form diacylglycerol (because we have two acyl groups and a molecule of glycerol). The last step is the esterification of the last hydroxyl group to form the triacylglycerol. Phosphatidic acid is the crossroad for the synthesis of membrane lipids.

SYNTHESIS OF TRIACILGLYCEROLS



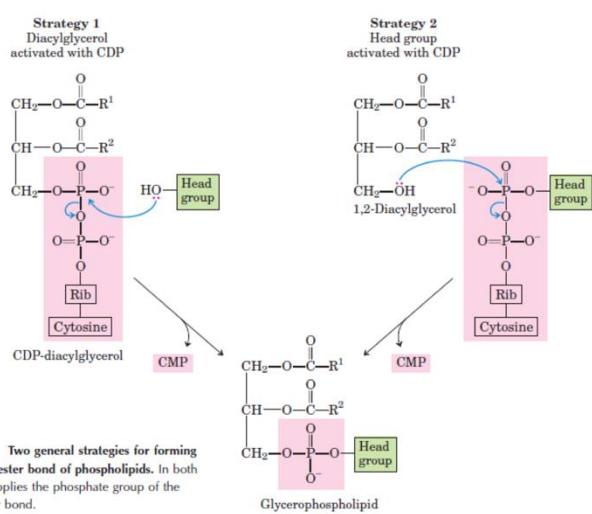
Structure and Polarity of A Glycerophospholipid



GLYCEROPHOSPHOLIPIDS

The glycerophospholipids are formed by a molecule of glycerol, two FA (a saturated and an unsaturated), a phosphate (the phosphatidic acid); the phosphate is linked to a polar head that usually derives from a molecule with an oxydrylic group, in this case this molecule is an alcohol because it has an oxydrylic group but often is cyrine (that is an aminoacid with an oxydrylic group, in this case we are concentrated on oxydrylic group and we consider cyrine as an alcohol but usually we consider it an aminoacid).

There are two different strategies for the synthesis of glycerophospholipids.



1st. We start from the Diacylglycerol (the first steps are the same from the triacylglycerol: we start from the glycerol in which we add the esterification of two FA, so we have the phosphorylation of the phosphate). Diacylglycerol can react with a molecule of CDP that gives the energy for the synthesis of the lipids (the energy for the synthesis of sugar, and in particular for the synthesis of glycogen, is UTP. For the synthesis of complex carbohydrates often the energy derives from UTP and for the synthesis of complex lipids, the energy derives from CDP). We have the formation of CDP-diacylglycerol, a molecule with a high energetic level; the phosphodiester link of phosphate gives the energy for the next reaction.

In fact we have our alcohol, our molecule with an hydroxyl group, and this hydroxyl group react with beta phosphate of our CDP and we

have the release of CMP (this reaction is an exergonic reaction) with the synthesis of glycerophospholipid.

2nd.

The synthesis starts from diacylglycerol and the activating molecule is an alcohol. The alcohol reacts with CDP to form CDP-alcohol; the reaction is similar to the previous one but the hydroxyl group that reacts with the phosphate is the hydroxyl group of diacylglycerol.

So there are two strategies:

- in the first one there is the activation of diacylglycerol with the CDP-diacylglycerol, so the energy derives from the CDP-diacylglycerol
- in the other one the energy derives again from the CDP group that is linked with the alcohol and not with diacylglycerol.

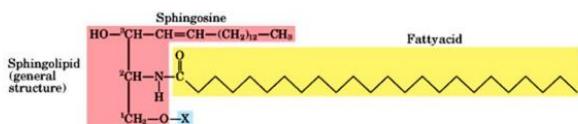
In this way, if one of the two enzymes of these reactions are mutated, we can still synthesize glycerophospholipids, because the other way is active.

These are some of the most frequent polar heads of glycerophospholipids.

Name of glycerophospholipid	Name of X	Formula of X	Net charge (at pH 7)
Phosphatidic acid	—	—H	-1
Phosphatidylethanolamine	Ethanolamine	—CH ₂ —CH ₂ —NH ₃	0
Phosphatidylcholine	Choline	—CH ₂ —CH ₂ —N(CH ₃) ₃	0
Phosphatidylserine	Serine	—CH ₂ —CH—NH ₃ COO ⁻	-1
Phosphatidylglycerol	Glycerol	—CH ₂ —CH—CH ₂ —OH OH	-1
Phosphatidylinositol 4,5-bisphosphate	myo-Inositol 4,5-bisphosphate		-4
Cardiolipin	Phosphatidyl-glycerol		-2

21-lipid synthesis and data analysis

SYNTHESIS OF SPHINGOLIPIDS



► Sphingolipids contain:

1. **Sphingosine** (shown in red), which is a long-chain amino alcohol: 18- carbon amino alcohol with C-C double bond
2. A **fatty acid** is joined to sphingosine via an amide linkage rather than an ester linkage as seen in glycerol.

Today we start with the synthesis of sphingolipids, that derive from a molecule that is called sphingosine. A chemical characteristic of sphingosine is that it is an amino alcohol and in fact here we have an amino group and an hydroxylic group. It is an hydrophobic amino alcohol because the last part of this molecule derives from fatty acids with long chains. Usually, this fatty acid is palmitic acid, a fatty acids with 16 carbons. The other part of sphingolipids is formed by a second fatty acid that is linked with the amino group by a peptide bond, that derives from the reaction of an amino group with a carboxylic group.

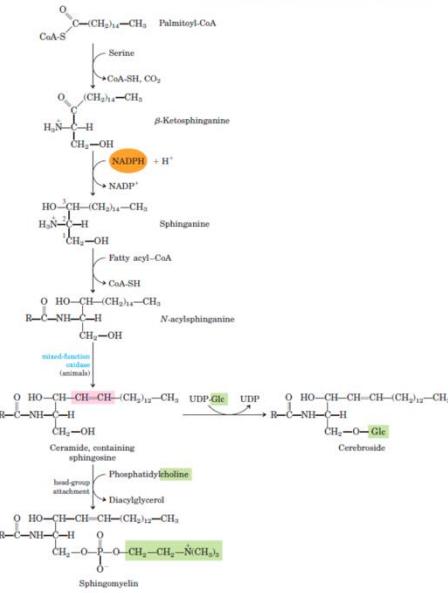
Then, the hydroxyl group can react with a phosphate and after with another molecule to form phosphosphingosine, and in this case we have a phosphosphingolipid. Otherwise, carbohydrate group can react with hydroxyl group forming glycosphingolipid (lipids without a phosphate group). In this case we don't talk about phospholipids because there isn't the phosphate. The general three-dimensional structure of sphingolipids is quite similar to the other glycerophospholipids because we have two hydrophobic long chain (derived from palmitic acid + other fatty acid), a

part that is similar to glycerol, a phosphate and a polar group. At the end the lipid has a polar head and 2 long hydrophobic tails. The organization inside the membrane is the same that we have seen for glycerophospholipids.

SYNTHESIS OF SPHINGOLIPIDS

The biosynthesis of sphingolipids takes place in four stages:

- Synthesis of the 18-carbon amine **sphinganine** from palmitoyl-CoA and serine;
- Attachment of a fatty acid in amide linkage to yield **N-acylsphinganine**;
- Desaturation of the sphinganine moiety to form **N-acylsphingosine** (ceramide);
- Attachment of a head group to produce a sphingolipid such as a **cerebroside** or **sphingomyelin**.



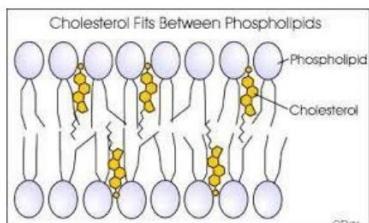
sphingomyelin, or adding different groups of sugars to form cerebrosides.

Cerebrosides are sphingolipids with different amount of glucose, and they are present for example in high concentration in membrane of neuron (the name cerebroside derives from the extraction of these sphingolipids from the membrane of the neurons). They are very important for the contact between neurons and as interactor between different parts of neurons, for connectivity and communication among different neurons.

In the structure of the membrane of these cells, usually cerebrosides are present essentially in the part that faces the extracellular medium, and the cerebroside are absent or less present in the part of the membrane that faces the cytosol, because they are important for the cell-to-cell contact.

SYNTHESIS OF CHOLESTEROL

Cholesterol has crucial role as a component of cellular membranes and as a precursor of steroid hormones and bile acids. All cells can synthesize it from simple precursors.



Cholesterol maintains the fluidity and increases the stability of the membranes. Without cholesterol the membrane would easily split apart.

The last part of the synthesis of lipids is the synthesis of cholesterol, the last type of lipid in membrane. Cholesterol is not a lipid like phospholipids or sphingolipids, because in this case we don't have the typical structure of a polar head and 2 long chains. Cholesterol is a little molecule (compared to phospholipids and other lipids in membrane), it has a very hydrophobic part in which we have several aromatic rings that are modified, and a little polar part in which we have an hydroxyl group, which interact with the polar head of lipids and the hydrophobic part interact with hydrophobic chain of lipids.

Considering that cholesterol is smaller than the other lipids, it is able to interact in the spaces between the different chains and in particular when fatty acid chains are unsaturated, so they are not linear but at different orientation.

The function of cholesterol is to maintain the fluidity of the membrane, that is important in order to avoid the freezing of our membrane, in particular when the temperature is low. Because of we have different fatty

acids that are saturated or unsaturated, the problem could be that the membrane can be separated in parts in which we have lipids that are in a solid state and another part in which lipids are fluid because they are more unsaturated. (Membrane fluidity is affected by fatty acids. More specifically, whether the fatty acids are saturated or unsaturated has an effect on membrane fluidity. Saturated fatty acids have no double bonds, and this decreases fluidity, making the membrane very strong and stacked tightly. Unsaturated fatty acids have at least one double bond, creating a "kink" in the chain. The double bond increases fluidity.)

Cholesterol interacting with different fatty acid chains act as glue in this way can maintain the fluidity and increases the stability of the membrane.

So, the synthesis of sphingolipids is quite difficult, I just want to show you the first important reaction because the sphingolipids derive essentially from molecules of palmitoyl coenzyme A. So, after fatty acids synthesis, a part of palmitoyl-CoA can enter inside the synthesis of sphingolipids.

Palmitoyl-CoA can react with an amino acid, serine. Metabolic pathway of synthesis of lipids and metabolic pathway of synthesis of amino acids are interconnected for the synthesis of sphingolipids. After we have several reactions (reduction, the addition of another fatty acid), at the end we have the core part of sphingolipid, that is ceramide.

Ceramide is a molecule in which we have the two long chain of lipids and hydroxylic group. This is already a sphingolipid because we have a polar head, that is the hydroxylic group, and 2 long hydrophobic chains.

From this molecule the other sphingolipids are created adding a phosphate, for example choline to form

The synthesis of cholesterol occurs in different organelles, but most of the synthesis of cholesterol occurs in cytosol.

However, the start of the synthesis is the condensation of 2 molecules of acetyl-CoA by the enzyme that is an isomer of the thiolase (it is the last enzyme in beta oxidation – if you remember this enzyme can catalyze the reaction of splitting of the last fatty acids in two molecule of acetyl-CoA).

There is an isoform of this enzyme also in cytosol, and it can catalyze the condensation of two molecules of acetyl-CoA to form acetoacetyl-CoA.

So, the first reactions are very similar to the reaction we have seen in the ketone bodies synthesis. The second reaction is the condensation of acetoacetyl-CoA with another molecule of acetyl-CoA. This reaction is catalyzed by the enzyme hydroxy-methylglutaryl-CoA synthase (this is an isoform of the protein that we have seen in mitochondria for the synthesis of the ketone bodies) to form a compound called hydroxy-methylglutaryl-CoA (or HMG-CoA).

Then, this molecule is reduced by 2 molecules of NADPH (molecule that can give electrons in anabolic pathways) to form a molecule with 5 carbons that is called mevalonate. This reaction is the reaction that committed the hydroxy-methylglutaryl-CoA to produce mevalonate and now the fate of mevalonate is to produce cholesterol. Therefore, this is the reaction that is strictly regulated in cells for the synthesis of cholesterol.

In fact, the enzyme hydroxy-methylglutaryl-CoA reductase is the enzyme that is the target for anti-cholesterol drugs. A lot of anti-cholesterol drugs acts inhibiting this enzyme that can reduce the synthesis of cholesterol in hypercholesterolemia.

SYNTHESIS OF CHOLESTEROL

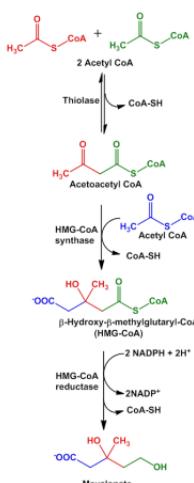
Stage 1: Synthesis of Mevalonate from Acetate

Two molecules of acetyl-CoA condense to form acetoacetyl-CoA

Acetoacetyl-CoA condenses with a third molecule of acetyl-CoA to yield the six-carbon compound β -hydroxy- β -methylglutaryl-CoA (HMG-CoA).

The cytosolic HMG-CoA synthase in this pathway is distinct from the mitochondrial isozyme that catalyzes HMG-CoA synthesis in ketone body formation.

The third reaction is the committed and rate-limiting reduction of HMG-CoA to mevalonate.



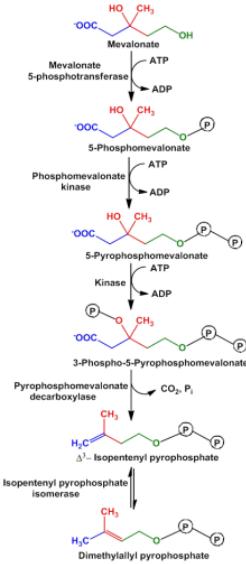
SYNTHESIS OF CHOLESTEROL

Stage 2: Conversion of Mevalonate to Two Activated Isoprenes

Three phosphate groups are transferred from three ATP molecules to mevalonate .

Decarboxylation of 3-phospho-5-pyrophosphomevalonate with production of a double bond.

Production of 3-isopentenyl pyrophosphate and isomerization in dimethylallyl pyrophosphate.



In the second stage of the synthesis of cholesterol we have the reaction that modify mevalonate (the starting molecule that is committed to produce cholesterol) to form the basic unit for the synthesis of cholesterol and for the synthesis of all isoprenoid lipids.

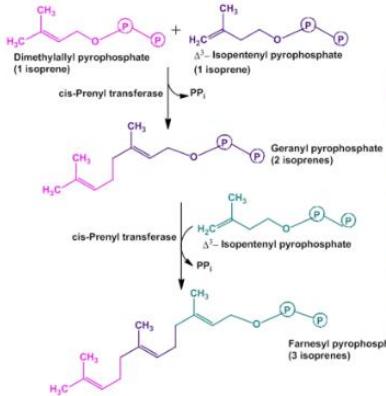
In fact, at the end of all this reaction we have the synthesis of an isoprenoid unit. Isoprene is a little hydrophobic molecule with 5 carbons and with 2 methyl that can be present in two isomers: isopentenyl and dimethylallyl. Each isoprenoid unit has characteristic to react tail-to-head or head-to-head to form polymers. For example, polymers in chemistry and polymers of isoprene can produce tissues for examples that derives from plastic (like pile) (isoprene can also be used to produce fibers for other polymerization and for different plastic materials). This because isoprene has the characteristic to react adding the same molecule several times to form long chains. So the synthesis of cholesterol of isoprene lipids starts from the formation of these 2 molecules: isopentenyl pyrophosphate (because

pyrophosphate activates the isoprene molecule – pyrophosphate group when is hydrolyzed release energy allowing the reaction to go in the direction of the synthesis) and dimethylallyl pyrophosphate.

To form this 2 molecules mevalonate is activated: 3 molecules of ATP are required to increase the energy of mevalonate, and then the energy of mevalonate is enough to allow the reaction of decarboxylation and dephosphorylation to form isopentenyl pyrophosphate and, thanks to isomerase, we can also synthesize dimethylallyl pyrophosphate.

In conclusion, the second stage of synthesis of cholesterol is the modification of mevalonate in the two isomers.

SYNTHESIS OF CHOLESTEROL



Stage 3: Condensation of Activated Isoprene Units to Form farnesyl pyrophosphate

Isopentenyl pyrophosphate and dimethylallyl pyrophosphate undergo a head-to-tail condensation to form geranyl pyrophosphate.

Geranyl pyrophosphate undergoes another head-to-tail condensation with isopentenyl pyrophosphate, yielding the 15-carbon intermediate farnesyl pyrophosphate.

The 2 monomers can react.

At the beginning head-to-tail reactions (in the head we have 2 methyl group): dimethylallyl pyrophosphate and isopentenyl pyrophosphate can react in a head-to-tail reaction.

After hydrolysis of the molecule of pyrophosphate (the reaction is exergonic) we have the formation of a molecule with 10 carbons (each isoprene have 5 carbons): this molecule is called geranyl pyrophosphate, that is the precursor of the smelling molecule of flowers.

Adding another molecule of isopentenyl pyrophosphate, we have the condensation of a third molecule yielding to the formation of farnesyl pyrophosphate in which we have 15 carbons (by addition of same molecules we can have polymerization and formation of this long unsaturated carbon chain).

The first stage of synthesis of cholesterol, two molecule of farnesyl pyrophosphate join head-to-head to form a molecule with 30 carbons.

This molecule is called squalene. The squalene is described in a structure that remember the final structure of cholesterol because we already have all the carbons that are required for the synthesis of cholesterol.

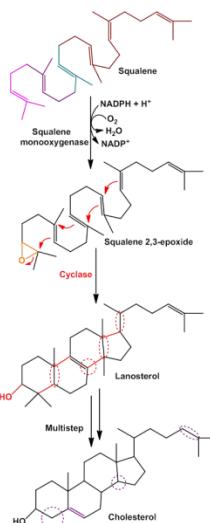
We have reduction from NADPH, oxidation to form hydroxyl group of cholesterol (oxygen is inserted in the molecule and then reduced to form hydroxyl group), and cyclization of squalene molecule to form the rings of cholesterol.

Cholesterol can be used for maintaining the stability of the fluidity of the membrane.

Lanosterol is finally converted in cholesterol in a series of about 20 reactions.

SYNTHESIS OF CHOLESTEROL

Stage 4: Conversion of Squalene to the Four-Ring Steroid Nucleus



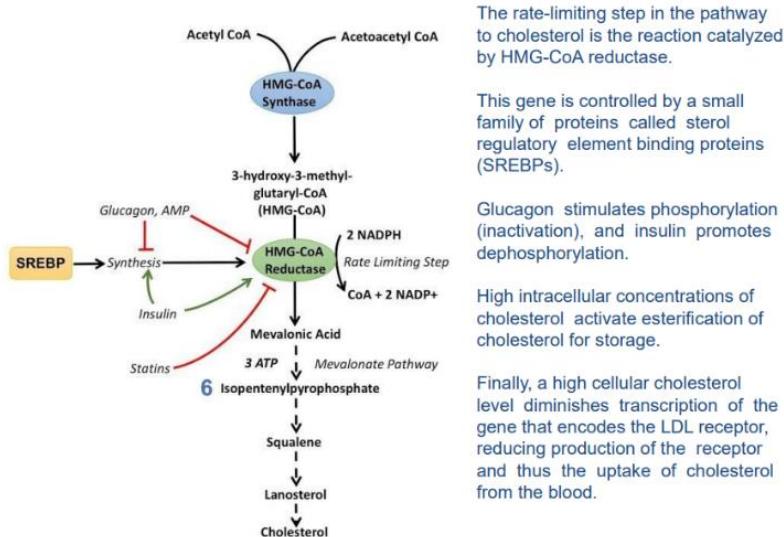
Two molecules of farnesyl pyrophosphate join head to head, with the elimination of both pyrophosphate groups, to form squalene.

The action of squalene monooxygenase adds one oxygen atom from O₂ to the end of the squalene chain, forming an epoxide.

In animal cells, this cyclization results in the formation of lanosterol, which contains the four rings characteristic of the steroid nucleus.

Lanosterol is finally converted to cholesterol in a series of about 20 reactions that include the migration of some methyl groups and the removal of others.

REGULATION OF SYNTHESIS



The rate-limiting step in the pathway to cholesterol is the reaction catalyzed by HMG-CoA reductase.

This gene is controlled by a small family of proteins called sterol regulatory element binding proteins (SREBPs).

Glucagon stimulates phosphorylation (inactivation), and insulin promotes dephosphorylation.

High intracellular concentrations of cholesterol activate esterification of cholesterol for storage.

Finally, a high cellular cholesterol level diminishes transcription of the gene that encodes the LDL receptor, reducing production of the receptor and thus the uptake of cholesterol from the blood.

This is the regulation of the synthesis of cholesterol. It is quite important, in fact cholesterol is not the only isoprene lipid produced in cells. From cholesterol several other hydrophobic molecules can be produced like hormones or coenzyme Q (isoprene chain of coenzyme Q that we have seen in respiratory chain derives from the first step of the synthesis of cholesterol).

The enzyme that is regulated is hydroxy-methylglutaryl-CoA reductase. This enzyme is regulated at allosteric level and at hormonal level. In fact, for example, AMP inhibits this enzyme because we have seen that the synthesis of isopentenyl pyrophosphate requires 3 ATP from mevalonic acid, so in new cells is important to have a lot of ATPs for synthesis of cholesterol. Moreover, from a hormonal point of view, glucagon is an inhibitor of the synthesis of an enzyme, the HMG reductase, while insulin is an activator of the synthesis – these hormones act at transcriptional level for

activating/inhibiting the synthesis of this enzyme.

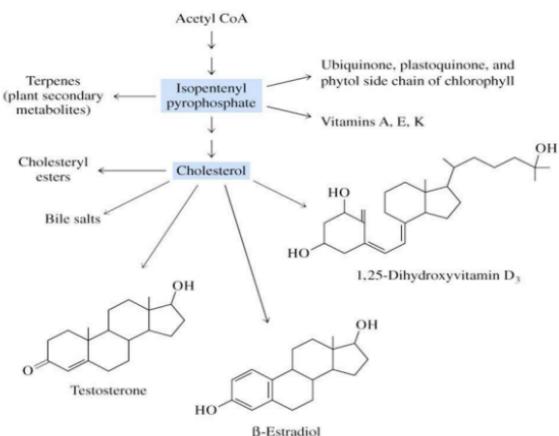
We also have proteins SREBP, that are proteins that regulate the activity of this enzyme – regulatory proteins that can induce the synthesis of the proteins and in particular of the synthesis of HMG reductase. The synthesis of SREBP is controlled by glucagon and

insulin. (Insulin and glucagon can act directly on the synthesis of HMG reductase activating different transcription factors, and in particular they can regulate the synthesis or activity of this protein that acts as transcription factors for the enzyme.)

One of the most famous drugs against the synthesis of cholesterol acts as an inhibitor of the HMG-CoA reductase. Some problems can occur during the use of statins, in fact in people that are particularly sensitive to the effect of statins there is not only the reduction of the synthesis of cholesterol but also the reduction of the synthesis of coenzyme Q, and some people can have collateral effects during the assumptions because the coenzyme Q is reduced in the synthesis. Therefore, people can have problems in mass (muscles are the first tissue that require energy for activity and sometimes people that used these drugs had problems with myalgia, fatigue) because the coenzyme Q is reduced and oxidative phosphorylation cannot properly occur in mitochondria.

The regulation of the synthesis of cholesterol is also at the level of receptors for cholesterol, in particular by the cells of liver. In liver there are receptors for lipoproteins – proteins that can transport lipids in blood. Some particular lipoproteins, like LDL or HDL, are transported for cholesterol and they are taken inside the liver cells by receptors. When we have a lot of cholesterol in our blood, these receptors take the lipoprotein but they are degraded so that the liver cells become insensitive to the ability to take LDL/HDL so that cholesterol remains in blood for example and cells limit the uptake of cholesterol in cytosol (but it is not good for our circulation because we have too much cholesterol in blood).

PRODUCTS OF CHOLESTEROL METABOLISM



pyrophosphate.

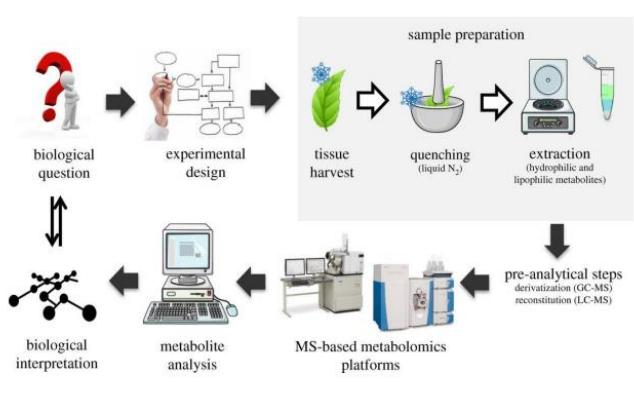
These are the different molecules that derive from the metabolism of cholesterol.

From the isopentenyl pyrophosphate we can have the synthesis of ubiquinone (=coenzyme Q) or plastoquinone in plants. Isopentenyl pyrophosphate is also precursor for the synthesis of chlorophylls, but it is also the precursor for the synthesis of vitamin A, E and K (we are not able to synthesize these vitamins, in fact they are called vitamins but in plants/bacteria these molecules are precursors of vitamins and in plants we also have other important hydrophobic molecules like terpenes, that derives from isopentenyl pyrophosphate. In addition, cholesterol is the precursor for other steroid molecules like hormones (like testosterone, estradiol...), vitamin D, bile salts, cholesterol esters (molecules that are transported in the blood to transport cholesterol in the tissues).

Important to remember that here we have HMG-CoA reductase, so all the mechanism that regulate this enzyme have an impact on all the molecules that derive from cholesterol and isopentenyl pyrophosphate.

GENERAL OVERVIEW OF THE MOST IMPORTANT METABOLISMS IN CELLS

GENERAL WORKFLOW

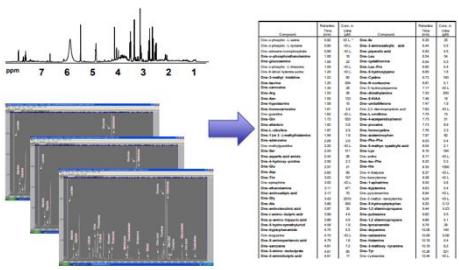


We have seen the importance of biological questions for metabolomics experiments, the experimental design, we have seen how the sample can be prepared, we have seen the most important technical analysis that are used in metabolomics that are NMR and mass spectrometry, and you have seen that mass spectrometry usually is coupled with pre-analytical steps that are essentially steps for separating molecules using chromatography, liquid chromatography and gas chromatography are the most used. You have seen the differences in NMR you can analyze directly your sample, but you can analyze in particular hydrophilic samples because the signal of water can be eliminated, but you need a high concentration of metabolites, because you can detect metabolites that are concentrated from micromolar and higher (no nanomolar).

On the contrary mass spectrometry is more sensitive but you have to manipulate your sample because we have to extract with different solvents, you have to separate by chromatography and then you have to volatilize your molecules... more steps in which you can lose molecules etc.

The 2 methods are both very used but, considering the biological question and the experimental design, you have also to choose which is the best method that you can use. Both are very expensive methods, so often it is difficult to have both the instruments in a lab, so often the analysis of metabolomics studies depends also on the instruments that you have.

FROM SPECTRA TO LISTS



Today we start to see how to manage data after the analysis.

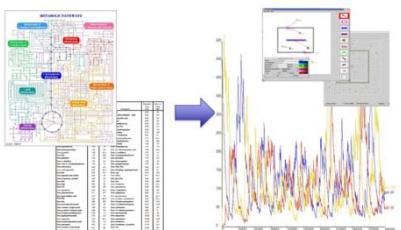
After the analysis we have some spectra of a mass spectrometry. You have seen how you can detect molecules from a spectrum, and, depending obviously from the type of analysis and from the spectra, you can obtain a list of metabolites and usually you can also obtain the concentrations of our metabolites (these derives both form mass spectrometry or NMR).

It is important that you know what the sources of the data is because the data analysis and the databases are different, so you have to consider the right data analysis.

what are, for example, the metabolic pathway that are activated/inhibited or that are involved in your metabolome analysis, because you have different molecules that are increased/decreased compared to samples. We want to see differences and do some biological interpretation of our experiment.

Final goal of the experiment is to analyze this data and have some info about the metabolism of your sample

FROM PATHWAYS & LISTS TO MODELS & BIOMARKERS



For example, in clinical analysis (but not only) analyzing the difference from controls and patient group you can identify some biomarkers/molecules that are specific in patients, and these are called biomarkers. So, from a metabolome analysis, at the end if you know which are the biomarkers you can say if your patient has the biomarkers for a specific disease or for another disease.

This is another goal of the analysis of the metabolome: to detect biomarkers.

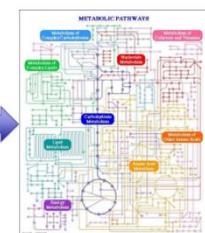
have to consider if your list of metabolites is good, so you have to check the integrity and the quality of your list of metabolites. Because, after all this step in which you control the integrity and the quality of your data, you perform statistic, and it is a very powerful method for analyzing data, but it is important that the starting group of data is good and correct, because it can give wrong results if you start with a wrong sample.

You have to check for inconsistency (means that if you perform the same experiment 2/3 times, if metabolites are presents only in 1 experiment and not in the other, they might be artifacts or there could be contamination in the sample, so you have to decide if data are consistent or not). You have to consider if you have some missing values, and sometimes you can miss values when you are at very high noise in the spectra, because if you see a lot of signals, you want to eliminate the noise and, in this case, sometimes you eliminate concentration in the noise. Again, is important to critically analyze these data additional signal not important in our sample. Usually, this type of analysis is performed by software but it is important to check, to have experience in the analysis of the data because sometimes you can remove some data or consider the noise as a data (experience is very important!). Software performs a lot of work but also the experience of scientist is very important.

In particular, these analysis of integrity and quality of data are important when you are performing mass spectrometry coupled with chromatography, because we have several steps, and we might have some problems during the steps and we have to consider that all the pipeline for the metabolome analysis are correct.

One important thing in this type of analysis is the peaking detection and the deconvolution of our peak. In this case are the peaks of the chromatogram: for each molecule separated by chromatography we can have a peak and its

FROM LISTS TO PATHWAYS



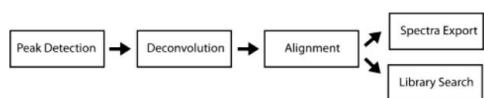
DATA INTEGRITY/QUALITY

- ❑ Once the data have been generated, the output has to be organized in a reasonable and intuitive structure.
 - ❑ Advanced technologies in metabolomics provide the opportunity of analyzing more than a thousand samples in an experimental run.
 - ❑ The complexity of the data need preprocessing and quality control steps prior to statistical analysis.
 - ❑ The quality control step is important:
 - To check for inconsistencies
 - To deal with missing values
 - To remove noises

DATA INTEGRITY/QUALITY

For LC/GC MS data preprocessing are:

- ❑ Peak picking detection and deconvolution → detection of each measured ion and assignment of a feature (m/z , RT pair).
 - ❑ Alignment → alignment of peaks considering slightly different retention time across the samples.



corresponds possibly to one metabolite, but usually it corresponds to 2 metabolites that are separated very close. We have to check if in these peaks there are one or more metabolites. This can be performed by peaking detection (individuation of the peak) and deconvolution of our peaks.

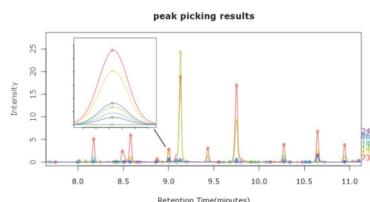
Another important thing is that, if we are performing the same experiment 2 or 3 times (in order to confirm our experiment we have to perform the experiment more than once) the chromatogram can have little differences because the temperature, for example, can modify a little bit the retention time of our elution of our molecules. So, it is important that when we are analyzing more than one experiment the chromatograms are aligned – we have to consider if our peaks corresponds in all three experiment for example (we have to align the chromatograms that might have some differences because the time of retention can have some shifts before or after comparing the other chromatograms).

At the end, we can analyze the spectra of each of our molecule and we can compare the spectra with a library to detect and to extract the info to know what our molecule is.

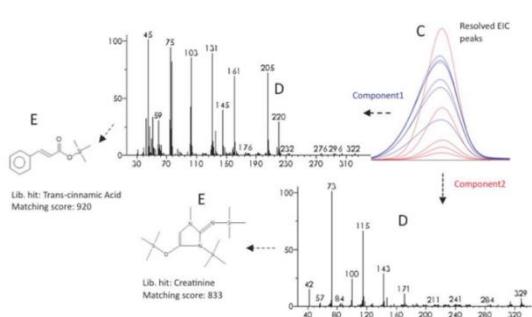
PEAK PICKING DETECTION AND DECONVOLUTION in LC/GS CHROMATOGRAPHY

In this step, the peak picking algorithm captures and deconvolves peaks from extracted chromatograms.

The first step of the workflow is to detect extracted ion chromatograms (EIC) peaks for each mass



PEAKS DECONVOLUTION



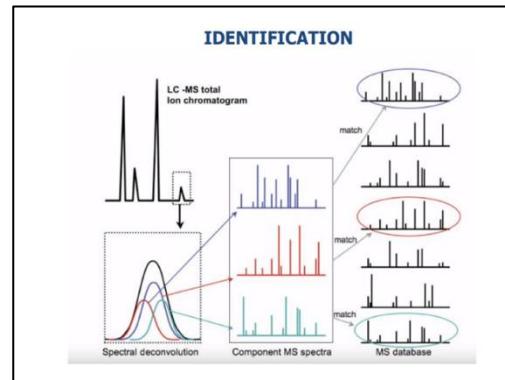
deconvolution to detect what is your molecule.

For example, the blue spectrum corresponds to the first spectrum, there is a perfect match. You can obtain the name of the molecule and eventually the concentration (remember that mass spectrometry is quantitative methods for analyzing metabolites).

This is another example. Here we have a peak that is not formed by only one molecule but from two, in which the blue fragments are in the same position of the peak and the red fragments are the other peak that is very very close and this correspond on the presence of two molecules.

Analyzing the fragments by mass spectrometry this is the real mass spectrometry spectrum, this is the signal corresponding to fragments that are present on the peak blue and this is the spectrum of the fragments that corresponds to the peak red. You can see that these 2 spectra can be separated and each of them can be analyzed to detect what is the molecule.

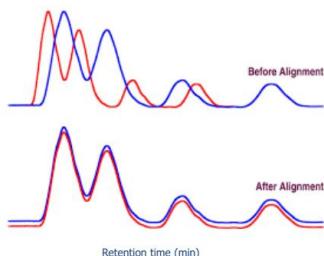
In order to do this, if you are lucky, you have a database of different spectra and you can compare all the spectra deriving from



ALIGNMENT

Because of differences in experimental conditions such as temperature and column conditions, the elution time that is observed for the same compound is usually shifted differently across samples and, as a result, alignment is needed to correct this shift.

Usually, alignment is performed on the total ion chromatogram spectrum and spectrum similarity algorithms are used to search for the same component across samples and to define a measure of confidence of the alignment obtained.



The last thing that you have to do is the alignment. If you are performing the experiment 2/3 times, this is important because replications are important in biological experiments. As I've told you before, the chromatogram, so the series of elution peaks, can be little bit different because the experimental conditions, the temperature and the column conditions might be a little bit different. So, if you have to analyze 2 or 3 chromatograms, you have to know if the red peak corresponds to the same molecule of the blue peak or if they are two molecules that are different in the samples.

So, you can use software or do it manually, you can try to align these two chromatograms and you see for example a little shift of the red chromatogram it perfectly overlap the blue chromatogram. The 2 chromatograms are the same, the samples have the same compounds, and it is good. The little difference is only due to elution time and retention time of your molecule depending on the experiment. If the overlapping is not present, you have to consider the 2 chromatographs completely separated and we have to consider that for example the red peaks correspond to a molecule and the blue peak to another molecule. On the contrary, if they are overlapping you have a replicate of your experiment and you can analyze each peak and analyze your molecule.

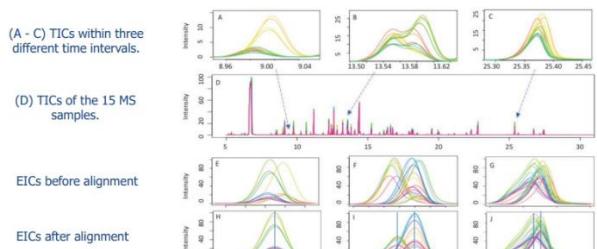
This is an example of an alignment of an experiment in which the author analyzes 15 mass spectrometry samples – each of these lines correspond to a chromatogram of an experiment. He did the experiment many times so that result is “very strong”.

Analyzing the concentration of the molecule in this peak for example are different but the presence of a peak more or less in the same position is present and after the alignment you see that this peak corresponds to only one molecule and in this case this peak corresponds to two peaks. Here again we have two peaks.

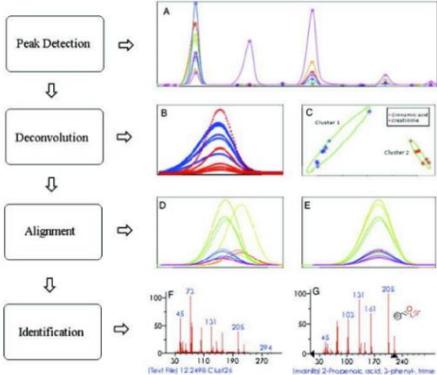
After this alignment the author analyzes each peak for each of the 15 samples, detect the molecules, and they can perform statistic. They have a very consisting data because they reproduced them 15 times.

ALIGNMENT

Alignment of three TICs from 15 MS samples with different concentrations



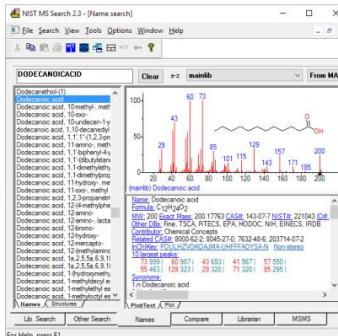
IDENTIFICATION



This is the summary of the part of the convolution of our experiment.

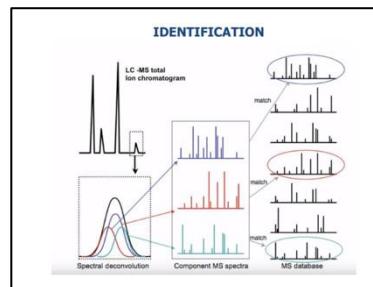
Here we have the peak detection, then the deconvolution in which we can analyze and we can see if we have different compounds, when we have more than one experiment we have to proceed with the alignment of our chromatograms and at the end we have the spectrum of our molecule and we can analyze in the database and match what is our molecule

IDENTIFICATION



The identity of a component for each peak is determined by searching the corresponding mass spectrum against a library of spectra.

Since peak area and peak height are directly related to the concentration of compounds in a sample, either of them can be used for quantification.

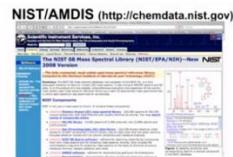


As we have seen, now we have software, and we can click on the spectra and the software review what is the spectra that matches the molecules. now we can know what are the molecules or what are the molecules that have similar spectra. Again, the experience of the scientist in this part is very important.

This slide only gives you an idea of how many databases for mass spectrometry or NMR. Obviously, they are different and are used to identify molecules.

Free databases. Usually when you buy mass spectrometer the company gives you some databases (not free), because these databases are selected for human metabolome, mammals metabolome...

MS SEARCH DATABASES



CFM-ID (<http://cfmid.wishartlab.com>)



NMR Software Packages (Alphabetical Order) Cited in this Review

Package	Source	URL
ACD	ACD Labs	http://www.acdlabs.com/resources/free/share/nmr_proc/
AMIX tool-kit	Bruker TopSpin	http://www.bruker-biospin.com/amix.html
Automics	Softpedea	http://www.softpedea.com/get/Science-CAD/Automics.shtml
CCPN Metabolomics	CCPN	http://www.ccpn.ac.uk/collaborations/metabolomics
Chemos NMR Suite	Chemonix	http://www.chemonix.com/software/
COLMAR	Florida State University	http://spinportals.magnet.fsu.edu/
dataChord Spectrum Miner	One Moon Scientific	http://www.onemoonscientific.com/dsm/summary.html
KnowItAll Metabolomics	BioRad Corporation	http://www.bio-rad.com.../KnowItAll-Metabolomics-Edition-Software
MetaboAnalyst	University of Alberta	http://www.metabolanalyst.ca
MetabolHunter	Natl Res Council of CA	http://www.archiveonfederates.ca/metabolhunter/
MetabolLab	University of Alberta	http://benegund.bham.ac.uk/mrmablab/d/metabolab.html
MetabolFitter	University of Alberta	http://wishart.biology.ubc.ca/metabolomex/
MNova	MetsterLab	http://metsterlab.com/software/mnova-nmr/
Newton	NMRFAM	http://newton.nmrfam.wsic.edu/
NMR-Pipe	National Institutes of Health (NIH)	http://spin.readthedocs.org/NMRPipe/
NUTS	Acorn Software	http://www.acornmr.com/nuts.htm
PRIME: SpinAssign	Platform for Riken Metabolomics	http://prime.pcr.riken.jp?action=standard_index
rNMR	NMRFAM	http://rnmr.nmrfam.wsic.edu
TopSpin	Bruker TopSpin	http://www.bruker-biospin.com/topspin1-dir.html
vNMRJ	Agilent	http://www.chem.agilent.com/en-US/products-services/Software-Informatics/vnmrj-30

Ellinger, et al., *Curr Metabolomics*. 2013

This is another list of databases for NMR. This list is old, now we have more databases.

Is important to choose the right database because when we are studying a specific biological sample is important to select the best database in which you can see your molecule.

For example, if you are performing a metabolome mammal/human cell is better to choose a mammal/human database and not a general database, because the metabolites of our body are different from other organisms.

PROBABILISTIC QUOTIENT NORMALIZATION

Quotient normalization assumes that the intensity of a majority of signals is a function of dilution only. Therefore, a most probable quotient between the signals of the corresponding spectrum and of a reference spectrum is calculated as normalization factor.

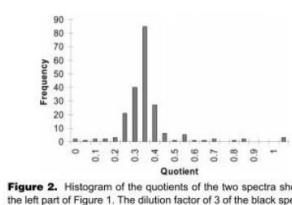
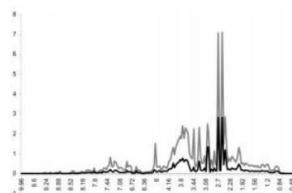


Figure 2. Histogram of the quotients of the two spectra shown in the left part of Figure 1. The dilution factor of 3 of the black spectrum is represented as the most probable quotient around 0.33.

Now we have the list of our molecules, the concentration of our molecules and before performing the statistical analysis is important to normalize our data because often we have some molecules that are present at very high/low concentrations, and we have to normalize our data in order to compare all these metabolites.

There are several ways to perform normalization.

Some methods for normalization are use of internal or external standards, in this case we have metabolites that are standard, so we know the concentration of our metabolites. In this way we can referred concentration and normalize the concentration of the molecules in our sample considering the standard curve that allow us to have a reference for the concentration of our molecule.

Another molecule is a total area normalization and we also have probabilistic quotient normalization and quantile normalization that are mathematical methods for normalizing our data.

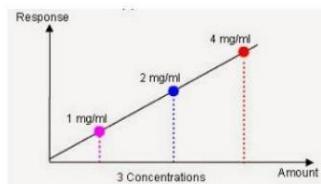
Now we see how they works.

ADDITION OF INTERNAL/EXTERNAL STANDARDS

- Used for targeted metabolomics.
- The samples can be normalized using the known concentration of an internal standard or performing an external calibration curve for a known metabolite.

The calibration curve is usually constructed by injecting an aliquot of the standard solution of known concentrations and measuring the peak area obtained.

After, the concentration of metabolites in the sample solution are extrapolated using the calibration curve



The addition of internal/external standards is very easy to understand.

In our sample, before the analysis, we can add a specific concentration of some molecules that are known. In this way we can analyze our sample and we can obtain a concentration of our metabolites that is referred to our standards.

In NMR we used standard TSP: concentration of TSP is known so the concentration of other metabolites are referred to this standard. This is an internal standard, in other cases we can do the same in mass spectrometry. We put inside to our sample some molecules with known concentration, and we can use them as internal standard.

If internal standard cannot be used, we can use external standards. Before the real experiment, we can analyze a solution in which we have our standard at specific concentration. We perform mass spectrometry analysis and detect the concentration, the height of peaks, and then we can create a calibration curve that can be compared with the metabolites in the sample.

TOTAL AREA NORMALIZATION

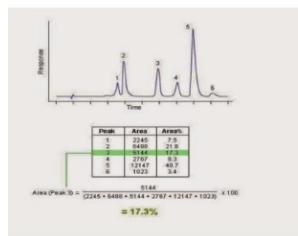
The normalization factor is the sum of the concentration of all metabolites.

The normalization factor could be affected by very different concentration among metabolites or by samples with different dilution

Usually the area normalization method reports the area of each peak as a percentage of the total area of all peaks.

The %A provides a suitable approximation of the relative amounts of components.

Limitations:
This method is applicable only when the peaks are similar
This method is applicable only for components eluted in a single run



The total area normalization is one of the most used method for normalization. In this case the amount of our metabolites is expressed as percentage of the presence of our molecule considering as 100% the total amount of the metabolites.

This is performed how? Considering the chromatogram (used often in mass spectrometry). The area of each peak in a chromatogram gives us the amount of our molecules. So, you can sum all the area of the peaks, so that you can have the total amount of your metabolites and you can express the percentage of each metabolites as the area of one molecule over the total amount of the metabolome.

In this case for example the compound 1 is present at 7,5% while compound 3 is present at 17,3% of the total area of your sample.

In this way you normalize the amount of all the metabolites considering the total amount of your metabolites.

There are some limitations.

For example, for each experiment you cannot express the percentage of metabolite obtained in another experiment considering the total amount of another experiment, because they are completely different. However, when you have the percentage, you can perform the experiment more times, and if, for example, your metabolites are always present at 7,5% of the total amount, now you can consider that is the real percentage of your metabolites.

This is another method, often performed in NMR. These are in fact an NMR spectra in which we can see spectra with several peaks. These two spectra, black and grey, are the spectra of two different biological sample. Spectra are similar, so the biological sample

are the same if metabolites are the same, but the amount of metabolites are different: one spectrum has an higher concentration than the other.

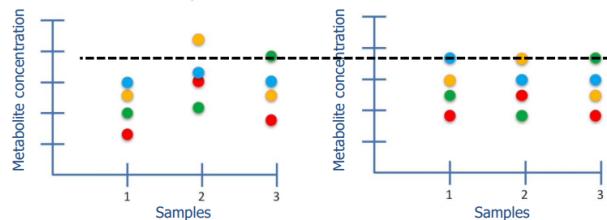
In this case we can normalize our data because we can consider how many times the more concentrated sample is concentrated corresponding to the other. In this example, more or less the concentration of the grey spectrum is three times as compared to the black spectrum, so we can normalize our data multiplying/dividing for three and we will obtain normalized data.

We can compare them because we are not interested in concentration of our metabolites but we are interested in the differences between the samples. In this way we have normalized the data and we can see the differences in the composition of metabolites of our two samples.

QUANTILE NORMALIZATION

This method forces all samples in a sample set to have identical peak intensity/area distribution. This method doesn't need of an estimated normalization factor.

This method can be problematic with high-value features in the data tables.



This last method is a mathematical method for normalization.

Again, we are not interested in the real concentration of our metabolites, but in normalizing them and know what are the metabolites more/less concentrates in our sample.

For example, here we have the first sample in which the blue metabolites is more concentrated than the red. In the second sample, the yellow metabolites is more concentrated than the green, and if you want to better analyze/normalize our data, using this method you can normalize our data maintaining the position (so the concentration) of our metabolites in each sample. After normalization we consider blue, yellow and green in the 3 samples that are the most concentrated and a media of our concentration is calculated (black line – medium value between blue, yellow and green). Now in the normalization all the metabolites are referred to the medium value but they are always the most concentrated in our sample. Performing the same for each values considering the order of concentration, at the end of normalization we will have data that are more similar as concentration, but again we are not interested in concentration, but we are interested in maintaining the order of concentration of our metabolites inside the samples. The most concentrated is the blue one in the fist and the less is the red one.

Now you can analyze these 3 samples and at the end of the statistical analysis probably you can say that there are differences among three group because there are differences in concentration of our metabolites depending on the sample. For example, the red one is the less concentrated in sample 1 and 3, but green is the less concentrated in sample 2. Analyzing the difference in concentration of the metabolites we can obtain data from statistical analysis of different groups (we can see if samples are different or overlapping).

DATA ANALYSIS

The avalanche of metabolome data presents great difficulties to analyze.

The problems in extracting meaning from large data sets are similar for all forms of profiling. The goal is to recognize patterns for further exploration.

METHODS OF DATA ANALYSIS

Univariate:
Fold change analysis
T-test
Volcano plots

Chemometrics:
Principal component analysis (PCA)
Partial least squares-discriminant analysis (PLS-DA)

Clustering:
Dendrogram & Heatmap
K-means Self Organizing Map (SOM)

Now we talk about what are the statistical methods that are used for the analysis of data.

When you want to compare two groups, if you want to see if there are difference between two groups, usually you use univariate statistical analysis, like T-test, fold change analysis and volcano plots. All these methods are important for analyzing differences between two groups.

Chemometrics analysis are statistical analysis that want to analyze several groups and wants to see if there are differences among the groups. We have a lot of data, and we have to use chemometric methods like (most used in metabolomics) principal component analysis (PCA) and partial least squares-discriminant analysis (PLS-DA).

The last type of data analysis is clustering in which we can obtain dendrogram and heatmaps, in which we can see for example whether a gene is expressed or not.

Univariate methods are the most used. We are analyzing two groups and we want to see if there are significant statistical differences between the two groups.

These are the three methods that we can use: the fold change (FC) analysis, student's t-test, and volcano plot, which is a combination of the first two methods and have a general overview of the most significant data.

UNIVARIATE METHODS

There are three methods available for univariate analyses:

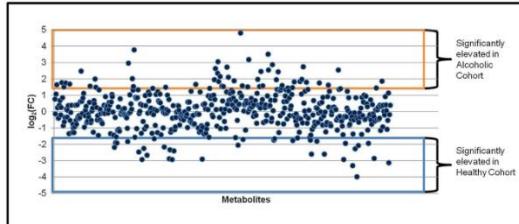
- Fold Change (FC) analysis,
- t-tests,
- Volcano plot which is a combination of the first two methods.

All three these methods support both unpaired and paired analyses.

They provide a preliminary overview about features that are potentially significant in discriminating the two groups under study.

UNIVARIATE METHODS FOLD CHANGE ANALYSIS

The fold change (FC) is calculated as the log transformation of the ratio between the mean metabolite abundance in the alcoholic cohort relative to the healthy cohort.



overrepresented in one of the two cohorts.

This is the fold change analysis the metabolomic experiment in which, as we can see in the example, metabolites are detected form the blood of a cohort of patient with alcoholic problems and a cohort of healthy patients. For each metabolite (each point is a metabolite) a concentration of this metabolite is obtained, and the ratio between the mean metabolite abundance in the alcoholic cohort relative to the healthy cohort. This ratio is called fold change. Some metabolites are present more in healthy cohort, while other are present more in alcoholic cohort. So, expressing the fold change as the logarithm in basis 2 of the fold chain we can obtain a graph in which 0 corresponds to the metabolites that doesn't change between the two cohorts, the positive number corresponds to metabolites that are more present/concentrated in alcoholic cohort with respect to healthy cohort, while in the negative there are metabolites that are more present in healthy cohort.

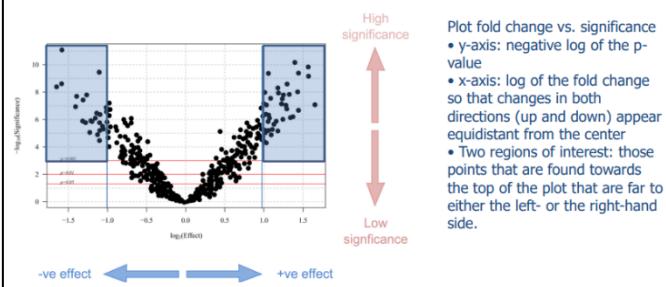
In this method are considered significant the metabolites that corresponds to three times more/less the fold change. We can see metabolites

The t-test can measure and analyze the difference between two groups.

Performing the t-test you analyze what is the medium values in your groups and after you can apply this equation in which is considered the differences inside each group and if the differences between the two groups are more than a specific value p we can say that the two groups are significantly different.

If p-value is less than 0.05, then that result is said to be statistically significant.

UNIVARIATE METHODS VOLCANO PLOT



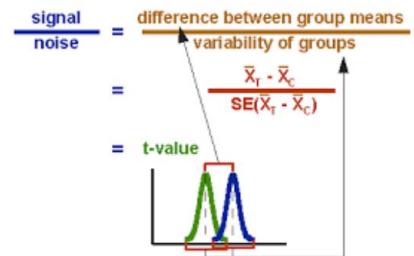
The volcano plot mix the two information provided by the fold change (x-axis, starting from 0 in which our metabolites are not different between the two groups) and t-test (y-axis, p value that we have calculated).

When the p value is very low, the value on y-axis is high.

In the shaded part of the graph we have the values of our metabolites that are the most significant different between the two groups.

In this case, these are the same data that we have seen for the alcoholic cohort, so these (left blue part) are the metabolites that are underrepresented in alcoholic cohort (negative) and here (right blue part) we have the metabolites that are overrepresented in the alcoholic cohort (positive). Using volcano plot we can see the two information deriving from the fold change and the t-test in the same graph.

UNIVARIATE METHODS t-tests



The t score is a ratio between the difference between two groups and the difference within the groups. The larger the t score, the more difference there is between groups. The smaller the t score, the more similarity there is between groups.

-NMR lecture (prof luca laghi)

NMR SPECTROSCOPY

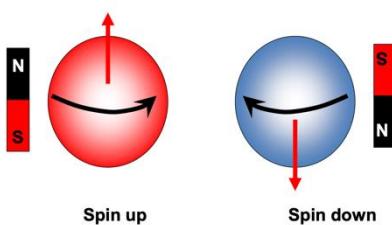
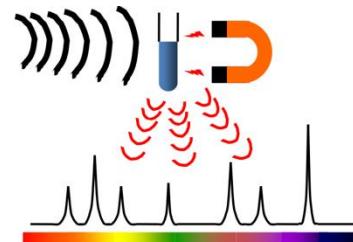
NMR can be considered as a universal metabolite detection technique, where samples can be analysed directly with minimal manipulation and many classes of small metabolites can be measured simultaneously. Major drawbacks in NMR for metabolomics are poor sensitivity and spectral complexity with superimposition of signals at certain spectral regions compromising clear identification

PRINCIPLES OF NMR

>NMR spectroscopy measures the absorption of radio waves due to changes in nuclear spin orientation

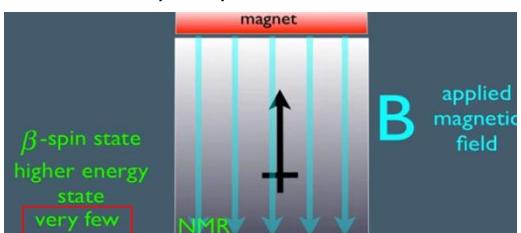
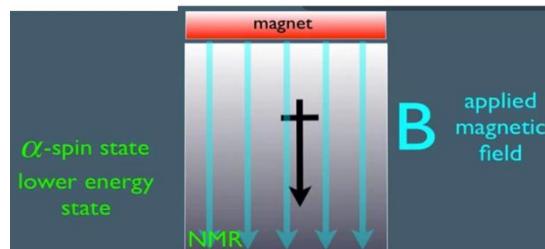
>NMR only occurs when a sample is in a strong magnetic field

>Different nuclei absorb at different energies (frequencies)

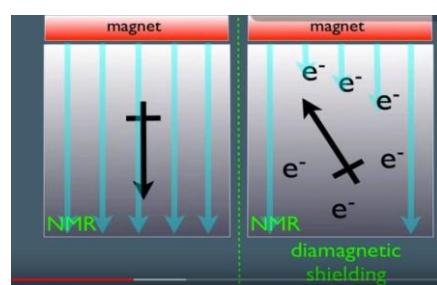


>Protons (and other nucleons with odd number of protons or odd number of neutrons) have a spin (1H, 13C, 15N, 19F, 31P)
>Each spinning proton is like a “mini-magnet”

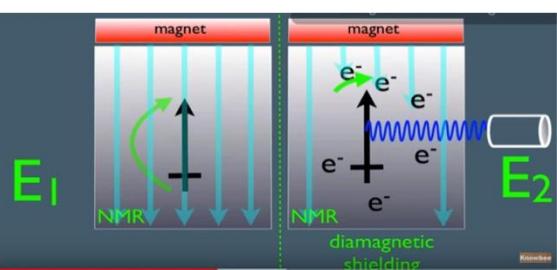
>When you put a magnet in a magnetic field it will orient itself with the magnetic field in a state of lower energy called alpha-spin state



>However this is not the only possible outcome here in fact it is also possible that the magnet could orient in this position in a state of higher energy called the beta-spin state



>A nucleus in a molecule could be surrounded by many electrons. In this case we talk about a shielded nucleus because electrons interfere with the magnetic field and protect nucleus from the effect of magnetic field



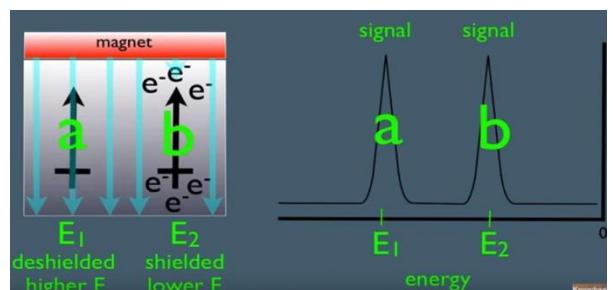
>If we give to this proton a light source that produces an RF radiation (radiofrequency radiation), it can happen that the energy is enough to move the proton in the B spin state

>E is the energy necessary to bring the proton in the B spin state.

>When protons are in this state we say that these atoms are in “resonance”

>When we put a molecule with two proton like the above mentioned in a NMR machine, we obtain a chart with two signals or peaks.

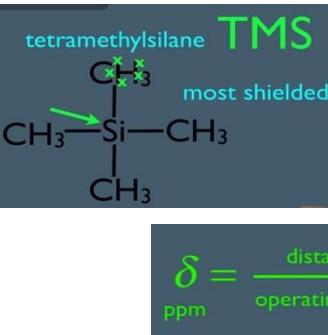
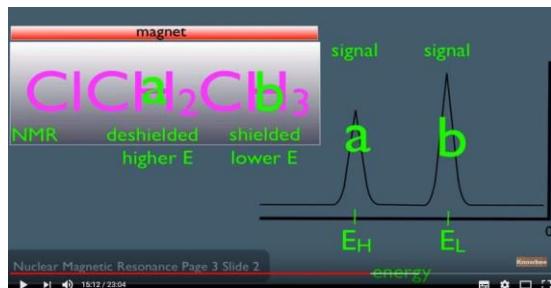
>Seeing two peaks means that you have two hydrogen atoms in different electronic environment.





NMR SIGNAL OF CHLOROETHAN

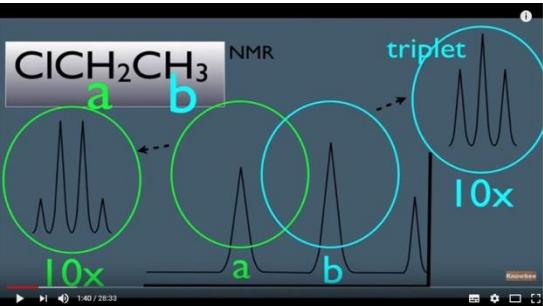
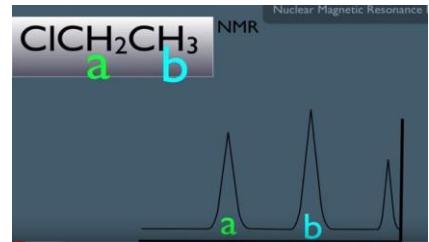
The number of peaks corresponds to the types of hydrogens. Proximity to an electronegative atom and/or multiple bond causes a peak to be shifted downfield



PEAKS SHIFTING

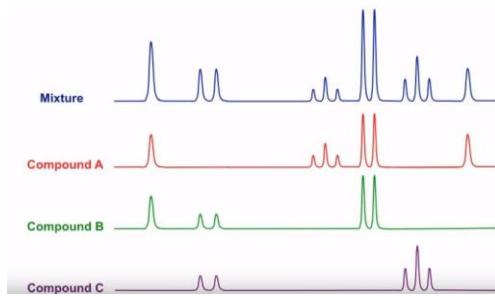
Tetramethylsilane is the reference molecule used for defining the peaks shifting because its protons are the most shielded .

proton	ppm
-CH ₃	0.85
-CH ₂ -	1.20
-CH-	1.55
=CH ₂	4.7
R-OH	2-5 variable



PEAKS CAN BE SPLIT DUE TO NEIGHBORING/DIFFERENT HYDROGENS

Hydrogens are influenced by the magnetic interactions between neighboring hydrogens giving a split of the signals. The number of split can be follow the rule N + 1 (N is the number of neighboring hydrogens)



WHAT DOES NMR MACHINE DO FOR US?

>Each compound can be defined by a unique pattern of chemical shifts (a fingerprint)

>If we know the molecules that we want detect, we can compare the spectra of each pure molecule with the spectra of mixture and find if our molecule is present in mixture.

>If we don't know what are the molecules in the mixture, we can compare a pre compiled data base of known molecules spectra and find which molecules are present in the mixture.>This procedure is called «spectral deconvolution»

NMR APPLICATION IN MEDICINE

NMR-based metabolomic techniques identify potential urinary biomarkers for early colorectal cancer detection (Wang et al., Oncotarget 2017). A total of 16 potential biomarker metabolites were identified in stage I/II CRC, indicating amino acid metabolism, glycolysis, tricarboxylic acid (TCA) cycle, urea cycle, choline metabolism, and gut microflora metabolism pathway disruptions.

Choline: lower urinary choline levels in CRC are most likely related to increased demand for phospholipid synthesis in tumor cells. Isocitrate: levels were reduced in CRC patient urine suggesting TCA cycle deregulation and increased energy metabolism due to tumor cell activation. Acetoacetate: levels are higher in CRC patients. Acetoacetate is a ketone body produced when cells need energy but TCA intermediate are insufficient. Glutamine: levels are higher in CRC. Glutamine is a precursor of alpha-ketoglutarate for TCA cycle.

3-fluxomics

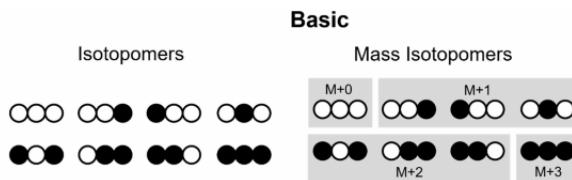
FLUXOMICS

> The complete set of metabolites is not enough to predict the phenotype, especially for higher cells in which the distinct metabolic processes involved in their production and degradation are finely regulated and interconnected.

> In these cases, quantitative knowledge of intracellular fluxes is required for a comprehensive characterization of metabolic networks and their functional operation.

> These intracellular fluxes cannot be detected directly, but can be estimated through interpretation of stable isotope patterns in metabolites.

> Mass fluxomics analysis (MFA) studies are carried out by feeding cells an isotopically labeled substrate (e.g., ^{13}C -labeled glucose) and subsequently measuring the patterns of isotope incorporation that emerge in downstream metabolites using mass spectrometry or NMR.



> Among

^{13}C -labelled substrates, those that have been most widely used as tracers are glucose labelled in the first or second position (i.e [1 or 1,2- ^{13}C]glucose) and the uniformly labelled glucose molecule ([U- ^{13}C]glucose). Depending on the metabolic pathway driven by the tracer, ^{13}C atoms are

incorporated into the newly formed metabolites in distinct numbers and at different positions.

> These isotopomers can be measured using liquid chromatography-tandem mass spectrometry or by NMR in order to know where and how the ^{13}C carbons are incorporated.

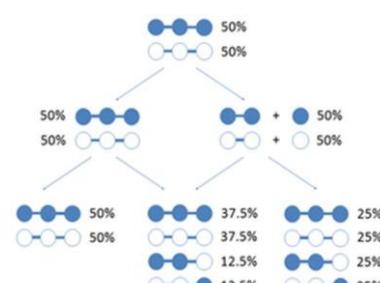
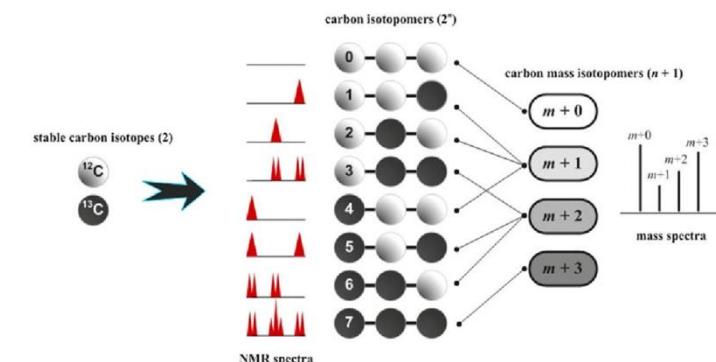
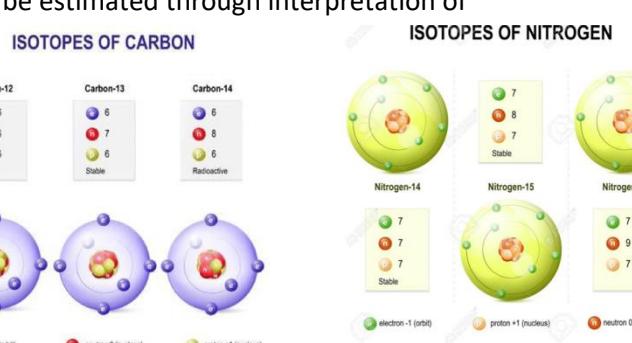
> A computational model of the intracellular metabolic network is used to determine pathway fluxes by integrating these isotope labeling data with additional measurements of extracellular nutrient uptake and product excretion rates.

In this way, metabolic flux analysis (MFA) can be used to reconstruct comprehensive flux maps depicting cell metabolism. If the various biochemical pathways inside the cell are considered as highways, MFA is analogous to generating a traffic report describing the flow through these highways and how they change in response to a roadblock or detour. Comparison of flux maps obtained under varying experimental conditions or in the presence of targeted genetic manipulations provides a functional readout on the global impact these perturbations have on cell metabolism.

> We can assume the original metabolite, a three carbon compound, has the ability to either split into a two carbon metabolite and one carbon metabolite in one reaction then recombine or remain a three carbon metabolite.

If the metabolite only takes the pathway down the left side, it remains in a 50–50 ratio of uniformly labeled to unlabeled metabolite.

If the metabolite only takes the right side new labeling patterns can occur, all in equal proportion. Other proportions can occur depending on how much of the original metabolite follows the left side of the pathway versus the right side of the pathway.



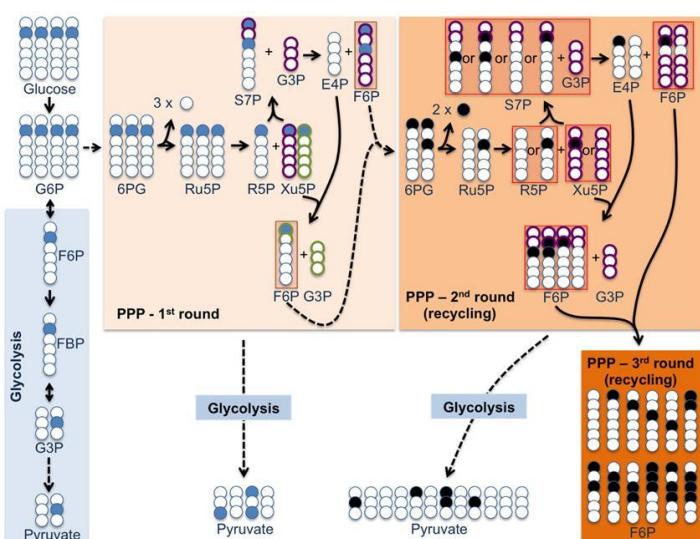
Here the proportions are shown for a situation in which half of the metabolites take the left side and half the right, but other proportions can occur.

>Once inside the cell, [U-¹³C]glucose is phosphorylated to [U-¹³C]glucose 6-phosphate, which can continue through the glycolytic pathway, producing glycolytic intermediates that are all uniformly labeled.

This entry of ¹³C-labeled acetyl-CoA results in two ¹³C carbons in all TCA cycle metabolites. Thus, M+2 isomers are isolated.

After the first turn, the resultant [1,2-¹³C]oxaloacetate or [3,4-¹³C]oxaloacetate can again condense with [1,2-¹³C]acetyl CoA.

After a given period of time we can measure different percentages of isotopomers that depend on the number of transformations (rate of reaction, regulation of enzyme activities etc..).



> Depending on the metabolic pathway driven by the tracer, ¹³C atoms are incorporated into the newly formed metabolites in distinct numbers and at different positions. Thus, for each metabolite there may be several isotopomers at different concentrations depending on the fluxes of metabolites in pathways.

> By measuring the isotopomer

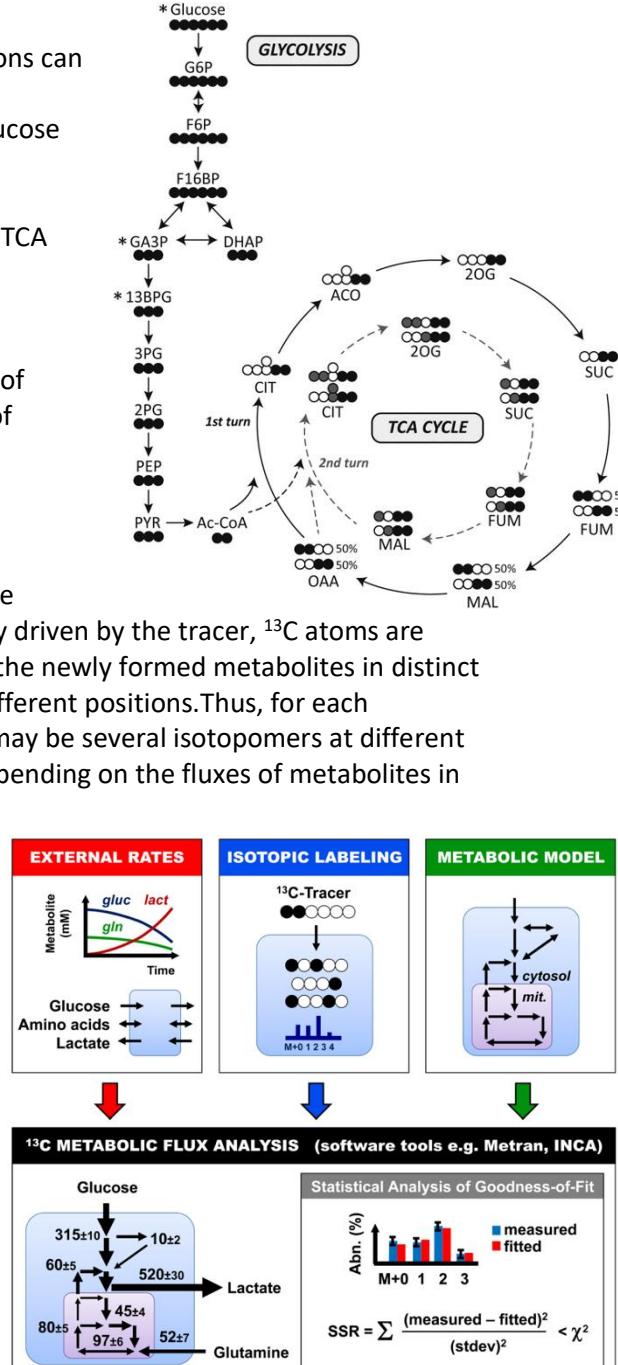
distribution of the differently labeled metabolites, the flux through each reaction can be determined.

MFA combines the data harvested from isotope labeling with the stoichiometry of each reaction, with the constraints, and with a theoretical flux map.

The irreversible reactions provide the thermodynamic constraints needed to find the fluxes.

The intracellular fluxes are estimated by using an iterative method in which simulated fluxes are plugged into the stoichiometric model.

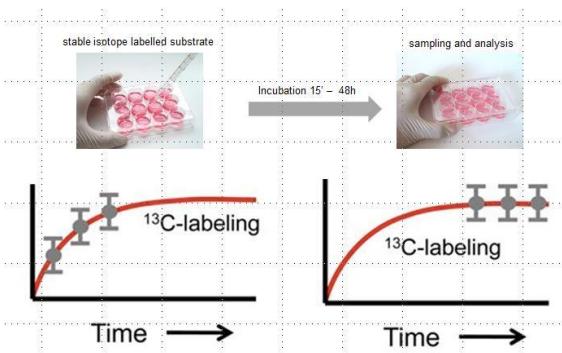
The simulated fluxes are displayed in a flux map, which shows the rate of reactants being converted to products for each reaction. In most flux maps, the thicker the arrow, the larger the flux value of the reaction.



> When conducting ¹³C-tracer experiments, a labeled substrate is introduced to the culture medium that is then taken up by the cells and metabolized through various metabolic pathways.

It takes a certain amount of time before intracellular metabolites reach a constant labeling state, which is referred as isotopic steady state.

The time required to reach isotopic steady state depends on the turnover rate of metabolites in a pathway and the labeling dynamics of upstream metabolites that feed into pathways.



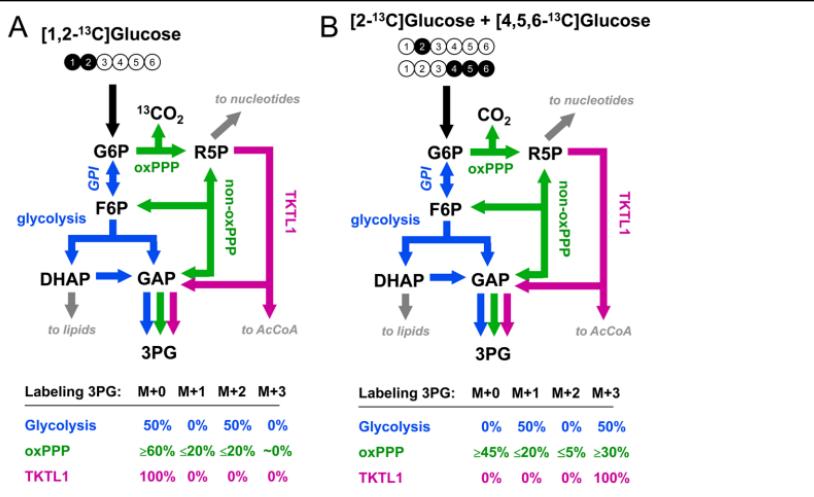
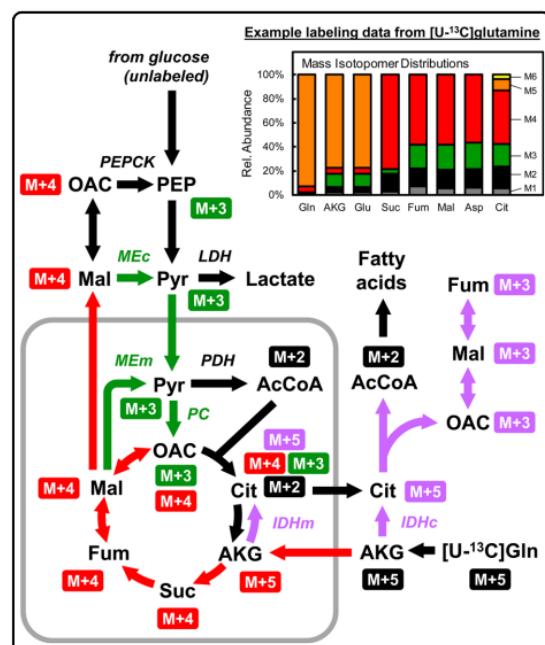


Fig. 6 Two alternative ¹³C-glucose-tracing strategies for analysis of metabolic fluxes in upper metabolism based on mass isotopomer measurements of 3-phosphoglycerate (3PG). **a** The [1,2-¹³C]glucose tracer allows good resolution of relative glycolysis and pentose phosphate pathway fluxes. **b** A mixture of 50% [2-¹³C]glucose and 50% [4,5,6-¹³C]glucose is an improved tracer approach that also allows precise quantification of the transketolase-like 1 (TKTL1) pathway flux

It is now well-known that there is no single best tracer for ¹³C-MFA studies. Generally, ¹³C-glucose tracers are best for determining fluxes in upper metabolism (e.g., glycolysis and PPP), while ¹³C-glutamine tracers typically produce better resolution of fluxes in lower parts of metabolism (e.g., TCA cycle and reductive carboxylation).



>[U-¹³C]Glutamine tracer experiments produce rich labeling patterns in TCA cycle metabolites that allow precise quantification of metabolic fluxes in lower part of central metabolism.

The diagram shows schematically the flow of ¹³C-labeling from [U-¹³C]glutamine into relevant metabolic pathways in cancer cells. The insert shows an example of labeling data obtained from a [U-¹³C] glutamine tracer experiment. Colors of arrows indicate different metabolic pathways: reductive carboxylation of glutamine (purple); glutaminolysis (red); conversion of malate to oxaloacetate via malic enzyme and pyruvate carboxylase (green).

CASE STUDY: A NEW GLYOXYLATE PATHWAY IN *S.cerevisiae* METABOLISM

The present case study will go through a series of metabolite analysis of yeast samples that began with general metabolite profiling of *S. cerevisiae* cultivated at different environmental conditions and ending with ¹³C-labeling experiments to confirm hypothesis raised from metabolite profiling data.

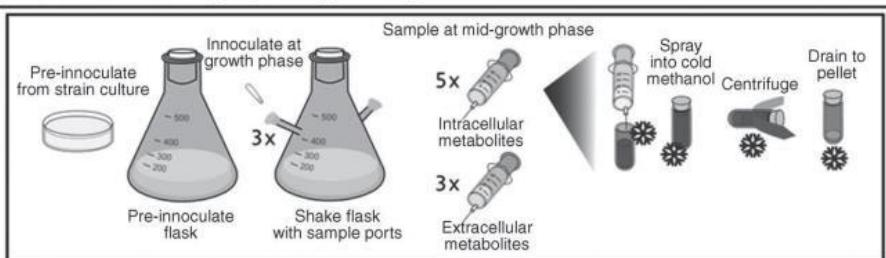
Hereby, we will illustrate how metabolomics alone can be a powerful tool to generate hypothesis that can be later tested using a more targeted approach.

Two strains of *S. cerevisiae* (laboratory strain and industrial strain) were cultivated in a standard medium (20g/l glucose) and in VHG medium (Very High Gravity – 250g/l glucose).

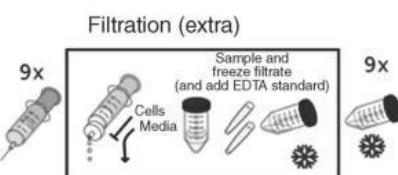
The samples for analysis of intracellular metabolites were harvested at mid-exponential phase using syringes, and quenched in non-buffered cold methanol solution (-40 °C).

The biomass was separated from the quenching solution by centrifugation at low temperature (-20 °C), and

Fermentation, sampling, and quenching



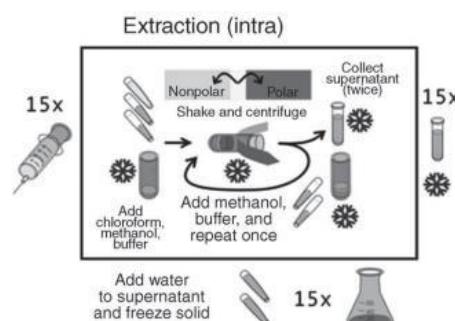
1 ml chloroform was added to the recovered pellet and stored at -80°C before metabolite extraction.



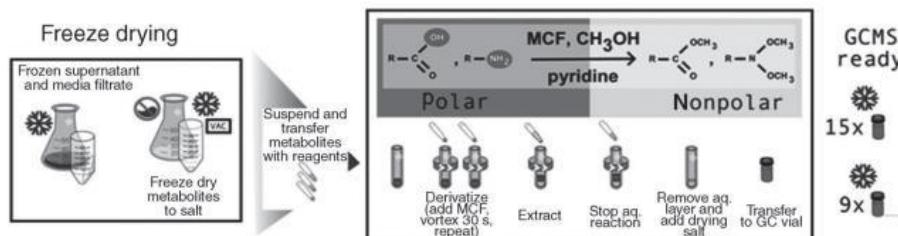
For analysis of extracellular metabolites the cell culture was harvested and filtered using Millipore membrane filters ($0.45\text{ }\mu\text{m}$) and the filtrated samples were stored at -20°C prior to analysis.

The intracellular metabolites were extracted from the cell pellets using a mixture of chloroform, methanol, and buffer at low temperature (-40 to -20 °C).

The mixture was separated into three phases (nonpolar, biomass, and polar) by centrifugation at low temperature (-20 °C). The polar phase was reserved for the analysis of the polar metabolites.



The dried samples were resuspended in sodium hydroxide solution and derivatized using methylchloroformate (MCF).



The metabolites were analyzed by GC–MS using a quadrupole mass selective detector, with electron ionization source operated at 70 eV.

Result: By applying principal component analysis of the data, glyoxylate appeared as an outstanding variable and, interestingly, inversely related to glycine levels. Both strains showed the presence of glyoxylate and glycine only in VHG medium.

Figure 1: Diagram of a quadrupole mass analyzer.

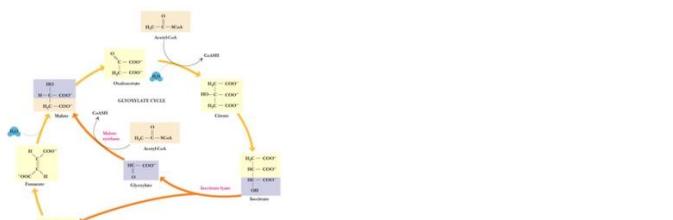


TABLE 6.1 Average of Intracellular Metabolite Concentrations ($\mu\text{mol/g}$ Dry Cell Mass) Obtained with the MCF Method and Calculated from a Total of Eight Independently Processed Samples (Devantier et al., 2005).

SD = standard laboratory medium; VHG = very high gravity fermentation medium

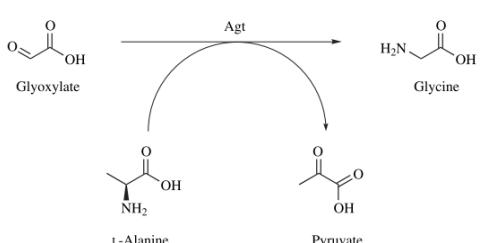
Glyoxylate should be not present in media containing glucose → isocitrate lyase is inhibited

Biological question: could glyoxylate derive through a metabolic pathway involving glycine?

Although this pathway was not described in *S. cerevisiae*, it exists in several microorganisms, e.g., *Bacillus subtilis*.

First hypothesis: glycoxylate derives from the deamination of glycine, which is used as nitrogen source.

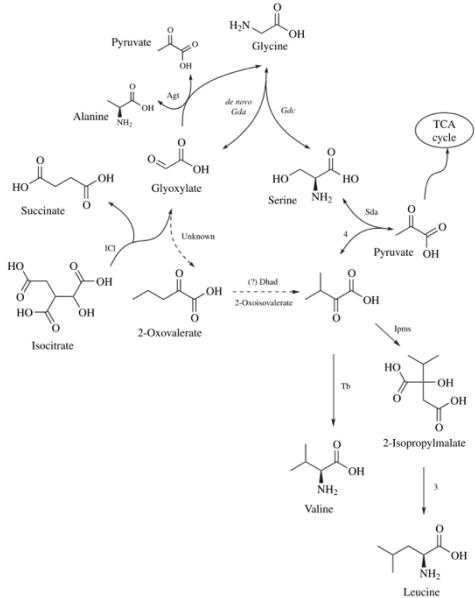
Agt: alanine:glyoxylate aminotransferase



Two strain: Agt wild-type and Agt deficient cultured in a medium with glycine as the only source of nitrogen and carbon. Result: both strains grow in selective medium → unlikely glycine is deaminated to glyoxylate.

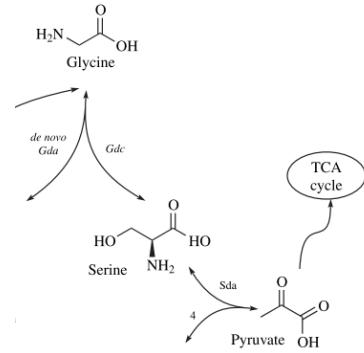
Second hypothesis: glyoxylate derives from of glycine decarboxylation and several other reactions that involve the production of serin, pyruvate, several TCA cycle metabolites and at the end glyoxylate

13C-(fully)-labeled glycine was used as the sole nitrogen source and its catabolism was followed by metabolite profile analysis of 13C-containing compounds using GC-MS
Result: serine, pyruvate and other TCA metabolites are not labeled → glycine is not decarboxylated to serine

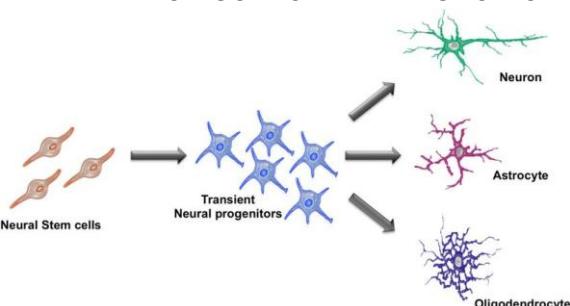


Conclusion: based on 13C labeled glycine it is clear that glycine can be directly oxidized to glyoxylate in *S. cerevisiae* following a new unknown pathway similar to the bacterial pathway in which an intermediate is 2-Oxovalerate.

A new glycine metabolic pathway in *S. cerevisiae* is proposed.
(S.G.Villas-Bôas et al. FEMS Yeast Research 5 (2005) 703–709)



QUANTIFICATION OF METABOLIC REARRANGEMENTS DURING NEURAL STEM CELLS DIFFERENTIATION INTO ASTROCYTES BY METABOLIC FLUX ANALYSIS



Biological question: how the central carbon metabolism evolves upon differentiation of neural stem cells (NSCs) into astrocytes?.

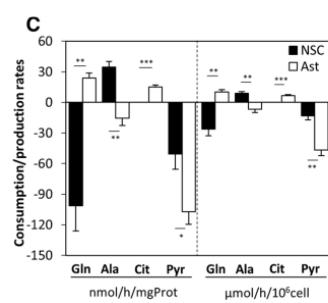
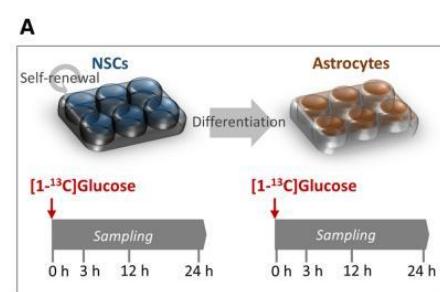
Why?: Because knowledge on the metabolic programs in NSCs and how they evolve during differentiation into somatic cells may provide novel therapeutic approaches to address brain diseases.

Astrocytes are the most abundant brain cells, being involved in virtually every function of the central nervous system, including energy metabolism, ionic homeostasis and synaptic transmission.

EXPERIMENTAL DESIGN

Murine embryonic stem cell (mESC)- derived NSCs and astrocytes were incubated with labelled [1-13C]glucose and the label incorporation into intracellular metabolites was followed by GC-MS.

The obtained 13C labelling patterns, together with uptake/secretion rates determined from supernatant analysis, were integrated into an isotopic non-stationary metabolic flux analysis (13C-MFA) model to estimate intracellular flux maps.



RESULTS

While glucose uptake was 1.7-fold higher in NSCs, a high lactate-secreting phenotype was common to both cell types. Furthermore, NSCs consumed glutamine from the medium while astrocytes secreted it. Another significant difference observed between these cell types was the secretion of citrate upon astrocytic differentiation.

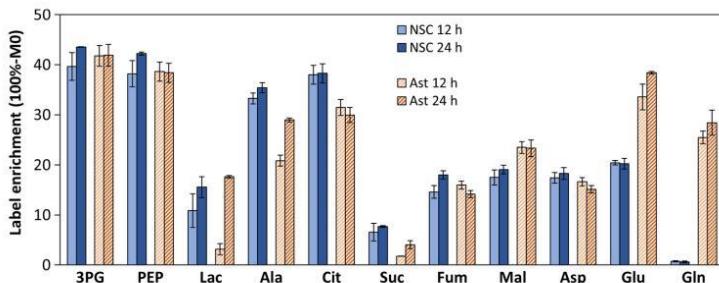
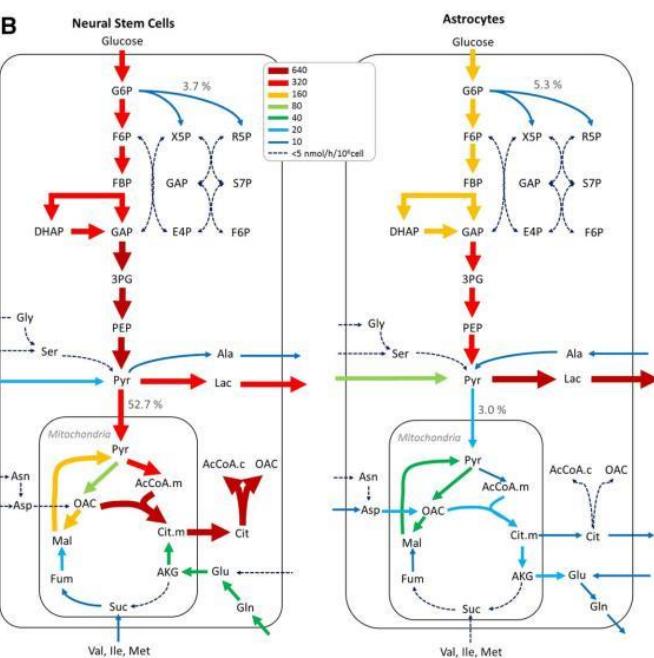


Fig. 2 Label enrichment of intracellular metabolites at 12 h and 24 h after [$1-^{13}\text{C}$]glucose addition to NSCs and astrocytes. The values correspond to the sum of all labeled isotopomers (100%-MO). Error bars represent the standard deviation of biological replicates. Shown

metabolites: 3-phosphoglyceric acid (3PG), phosphoenolpyruvate (PEP), lactate (Lac), alanine (Ala), citrate (Cit), succinate (Suc), fumarate (Fum), malate (Mal), aspartate (Asp), glutamate (Glu) and glutamine (Gln)

label enrichment in lactate, alanine, TCA cycle intermediates and related amino acids, indicating the contribution of other carbon sources.



METABOLIC FLUX MAPS FOR NSCs AND ASTROCYTES.

NSCs displayed a higher metabolic flux in central carbon metabolism compared with astrocytes, including glycolysis and the TCA cycle.

These flux maps show that the activity of the PPP remained lower than 6% of the corresponding glycolytic flux in both cell populations.

Half of the cytosolic pyruvate was converted to mitochondrial pyruvate in NSCs, while in astrocytes most of the cytosolic pyruvate was diverted to lactate.

In mitochondria, the majority of pyruvate in NSCs entered the TCA cycle by conversion to Acetyl-CoA through pyruvate dehydrogenase activity and the rest was carboxylated to Oxaloacetate. Glutamine was taken up from the medium by NSCs and metabolized to citrate via reductive carboxylation of alpha-ketoglutarate (AKG). The importance of this metabolic route was first identified in cancer cell cultures to support lipogenesis in cells with high

growth rates. On the other hand, astrocytes metabolism of pyruvate through PDH and lactate is secreted. The rest of pyruvate enters in TCA cycle but alfa-ketoglutarate is aminated to Glutamate and after to Glutamine that is secreted

CONCLUSIONS

NSCs consume glucose and glutamine from the medium.

Glucose is used in part for producing ATP and in part for producing citrate.

The highly active reductive carboxylation of alpha-ketoglutarate indicates that this is converted to citrate and used for biosynthetic purposes.

NSCs have a high anabolic metabolism proper of cells in active division.

In astrocytes, pyruvate entered the TCA cycle to produce citrate and alfa-ketoglutarate.

This pathway supported glutamine and citrate secretion, which are important metabolites for the proper functioning of other cells in the nervous system.

In both cell populations, the label enrichment of the glycolytic intermediates 3PG and PEP reached values over 40%, showing conservation of the ^{13}C label coming from glucose. Since the first carbon of glucose is lost as CO_2 in the oxidative branch of the PPP, these results indicate that the non-oxidative PPP branch had low activity in both cell populations. Overall, the

