25

Faculty of medicine – JU2018

◉ Sheet ○ Slides

DONE BY

Batool Albdour

CONTRIBUTED IN THE SCIENTIFIC CORRECTION

Deema Noaman

CONTRIBUTED IN THE GRAMMATICAL CORRECTION

Deema Noaman

DOCTOR

Ma'moun

Notice: this sheet was written according to dr.Ma'moun's lecture, this sheet will continue talking about cofactors which started in the previous, as well as start talking about protein purification techniques. :D

Enzymes will be underlined.

Quick recap: in the last lecture we have started talking about cofactors and here are some important things you should know by now.

i. cofactors are nonprotein groups that provide help for the enzymes to do their work.
ii. cofactors are classified according to their chemical structure into (metal, small organic molecules and protein bases)
iii. coenzymes are organic molecules.
iv. the most important coenzymes are vitamins and some of their derivatives (especially vit. C and B).
v. our body converts inactive vitamins into their active forms so they can perform their biological function, mostly by modifying them.
vi. Thiamin (active: thiamin pyrophosphate) is important for decarboxylation reactions.
vii. Biotin (vitamin B7) is important for carboxylation reactions.
viii. an apoenzyme is an inactive conjugated enzyme because it isn't connected to a substrate nor a cofactor.
ix. a conjugated enzyme is an enzyme that needs a cofactor to function.
x. apoenzyme + (cofactor and substrate) = holoenzyme

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* We will start this sheet by talking about :

oxidation- reduction coenzymes.

-These coenzymes actually work alongside (within) oxidoreductases, they can accept or transfer electrons in the form of hydrogen (ions / atoms) or oxygen.

-Each coenzyme has a unique functional group that accepts and donates electrons and is specific for the form of electrons it transfers (e.g., hydride ions, hydrogen atoms, oxygen).
-oxidation-reduction coenzymes do not form covalent bonds with the substrate, only a portion of the coenzyme binds the enzyme.

- Two of the most important(and most common)cofactors of this type are:
a) NAD (niacin, B3).
b)FAD (riboflavin, B2).

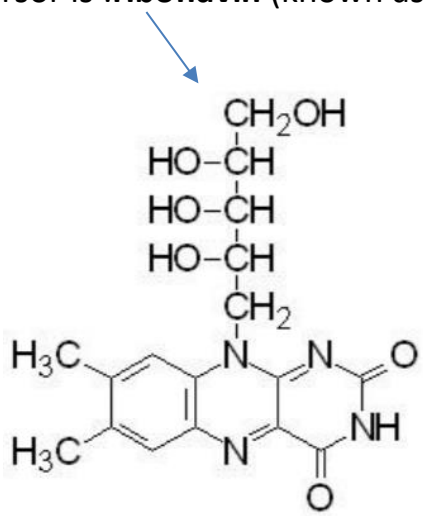
- Some oxidation-reduction coenzymes work with metals to transfer single electrons to O2 (Vitamins E & C)
-Their **specificity** and catalytic power depends on the enzyme that they bind to.

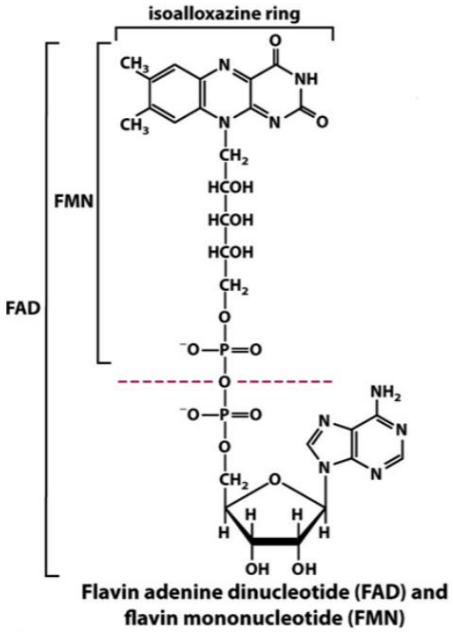
1.FAD and FMN :

a. BOTH are prosthetic groups of flavoproteins.

*remember: they're tightly bound to enzymes.

b. Its precursor is :**riboflavin** (known as **vitamin B2**)





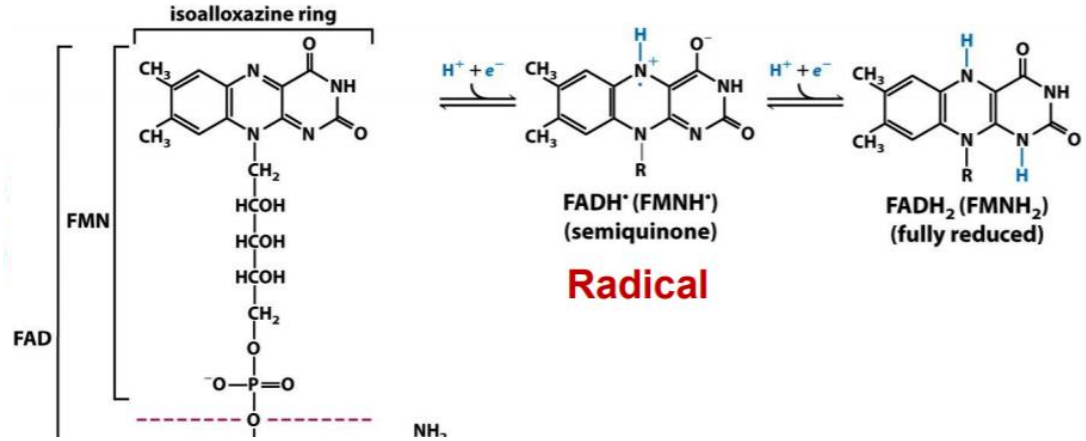
Flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN)

***Why** is it considered a prosthetic group?

**It being a prosthetic group is a protective mechanism, because electrons are transferred to FAD through the nitrogens of flavin structure itself sequentially, meaning one by one. This sequential transfer of electrons creates radical

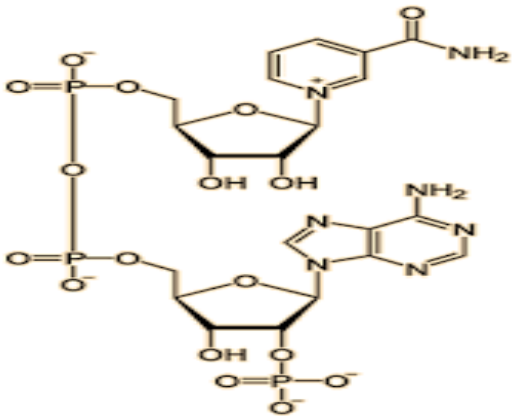
intermediates that are tightly attached to the enzyme (since it's a prosthetic group), but if they were loosely attached (not prosthetic), they would dissociate from the enzyme and attack other molecules thus damaging cells or tissues.

c. pyruvate dehydrogenase and succinate dehydrogenase:



require FAD as a cofactor and are important for kreb's cycle.

2.NAD (nicotinamide adenine dinucleotide.)



a. another form is NADP+ , the difference between them is that NADP+ has a phosphate group, which is only Important for enzyme binding and to specify the enzymes that need NAD or need NADP.
-These are cosubstrates for numerous dehydrogenases

*in the metabolism course we'll see that :

1) NAD is needed for catabolism, degradation reactions, oxidation reactions like oxidation of glucose into pyruvate to enter kreb's cycle.

2)NADP is needed for anabolism (building of larger molecules).

b. their precursor is **vitamin B3** known as **niacin**.

c. they're cosubstrates for common dehydrogenases.

Mechanism of how NAD works:

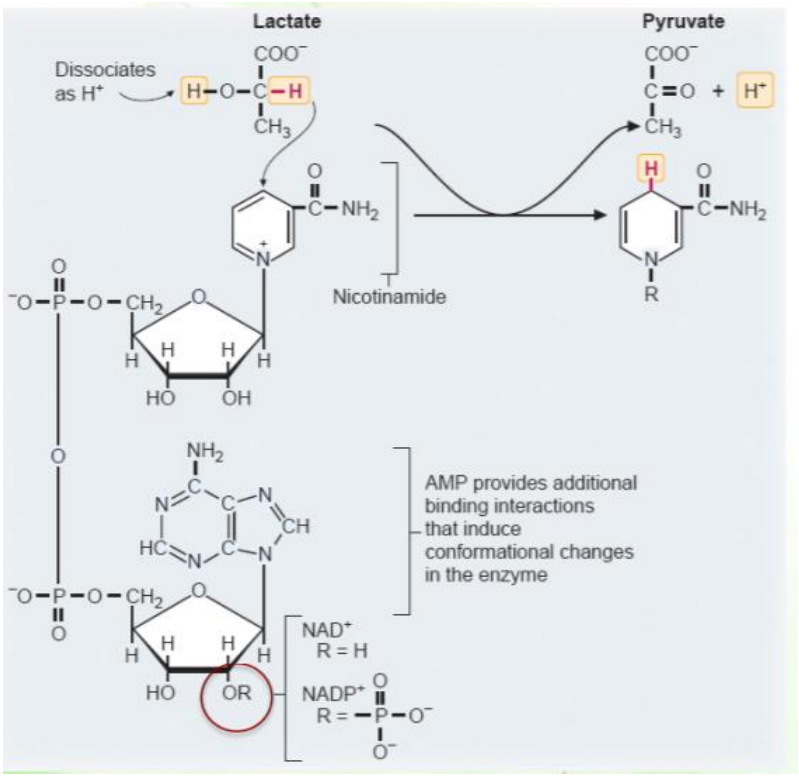
The cofactor accepts a hydride ion from the substrate, dissociates, & a keto group (CO) is formed.

Note: electrons are transferred to NAD in the form of **hydride (H-)**.

Also note that: the functional group is the C opposite to N.

For example we'll use the transformation from lactate to pyruvate:

This hydride (from lactate) dissociates and is transferred to NAD making it NADH and a keto group is formed on the lactate transforming it into pyruvate by lactate dehydrogenase.



The reverse reaction is catalyzed by **the same enzyme**, but in this case NADH acts as the source of the electron. In which the hydride ion is transferred to the ketone group forming a hydroxyl.

Pyruvate → lactate = reduction | lactate→ pyruvate = oxidation

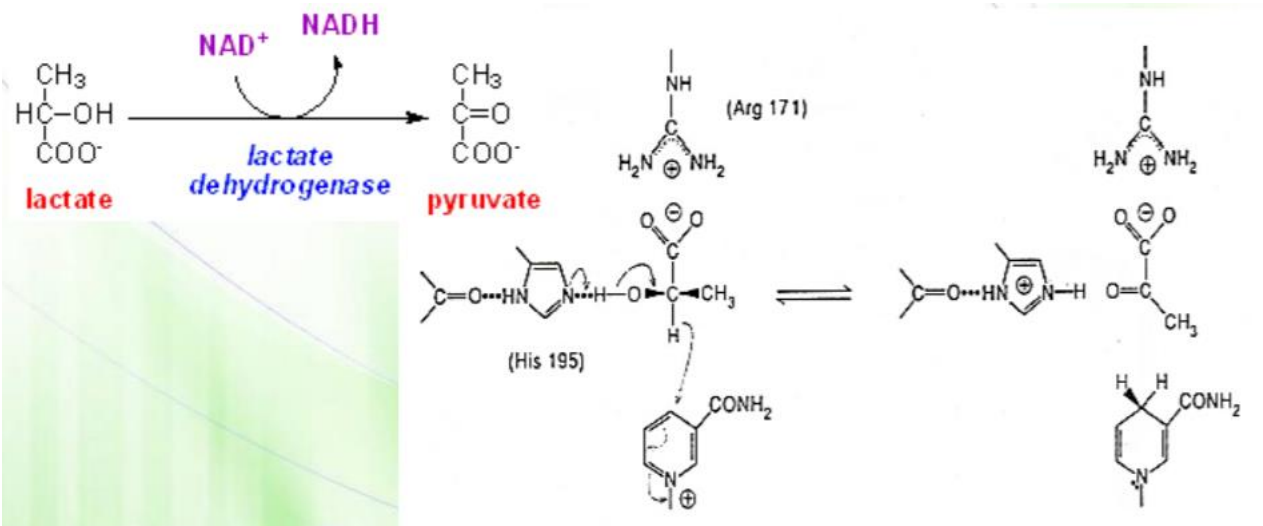
-the differences between NADH and FADH2 are in terms of what group is accepted and in terms of their interaction with enzymes:

FADH2 is a prosthetic cofactor because it binds tightly to the enzyme.

while, **NADH** and **NAD** are known as cosubstrates, because they bind loosely to the enzyme, and because of that there's no danger from NAD because it moves both electrons to the same group at the same time, so there's no radical intermediate.

An enzyme that uses NAD is **Lactate dehydrogenase**, in the active site of this enzyme we have a histidine that binds to the protons of(-OH) on lactate making it easier for NAD+ to pull off the other hydrogen with both electrons (a hydride).

So, **Histidine** makes it easier for the electrons to be transferred from lactate to NAD+, so we have a cooperation between the active site of the enzyme and the cofactor that works alongside it to move electrons from one molecule to another.

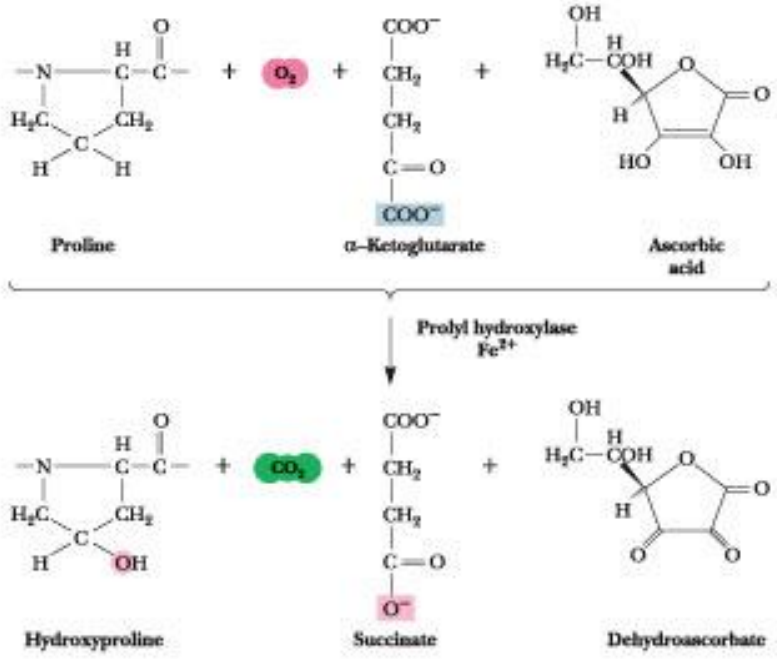


Vitamin C (ascorbic acid):

It is a :

1) cofactor:

It is used as a cofactor in hydroxylation of proline, which is found in collagen. (it's important for the function of prolyl hydroxylase, the enzyme that forms **hydroxyproline** which is a distinctive residue of collagen)



2)antioxidant:

It's also an antioxidant because it donates electrons to oxidizing agents and it gets oxidized itself.

Please note that in what we're discussing next, the mechanism is what matters, the structures don't:

-Reactive oxygen species oxidize (take electrons from) ascorbate into a radical itself, which is then oxidized into dehydroascorbate. (the functional molecule of vitamin C is known as **ascorbate**.)

-The structure of vitamin C (and other anti-oxidants) is preferable due to formation of resonance. (we have a ring structure as part of the vitamin, which is an advantage because there's a formation of **resonance structures**, due to movement of bonds.) Therefore, the oxidized forms of ascorbate are relatively stable, unreactive, and do not cause cellular damage.

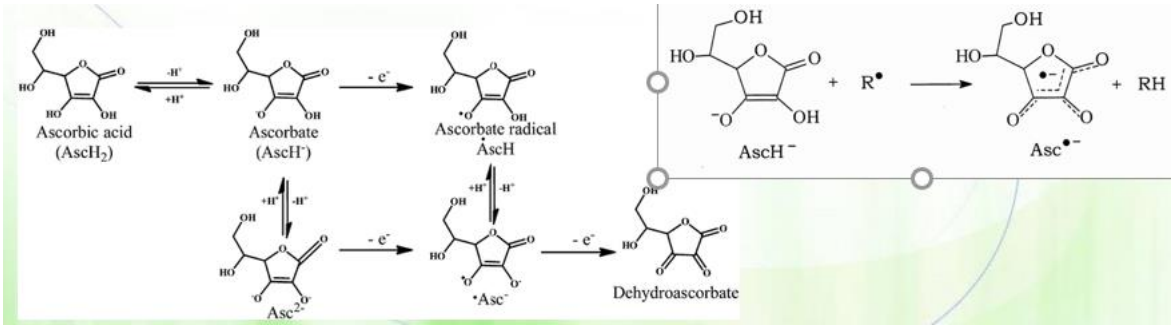
Imagine that we have a chain, if one electron was accepted (so we have formation of a radical group) and this electron keeps moving from one group to another until it reaches the end of the chain it moves in and then it has to jump and go somewhere else, it attacks another molecule damaging it , so the advantage of this ring structure is that this electron will be accepted by one group and it keeps on moving in a circle and it doesn't jump somewhere else, it's kept imprisoned in this ring structure.

Having a ring is a protection mechanism, that's why it's important having antioxidants in the form of active ring structure.

METALS:

Some Enzymes need metals to function, like carbonic anhydrase.

Hexokinase needs **magnesium**, remember when we talked about vitamin B1 , the process of **chelation**, this magnesium binds to the active site in the enzyme and is needed for the very first reaction in the metabolism of glucose to glucose-6-phosphate.



These metals function as **electrophiles**, they tend to extract electrons from other molecules.

Metals can:

Metal	Enzyme
Zn^{2+}	Carbonic anhydrase Carboxypeptidase
Mg^{2+}	Hexokinase
Se	Glutathione peroxidase
Mn^{2+}	Superoxide dismutase

1. bind to substrates and stabilize the formation of anions

2. stabilize the intermediate

3. help in the formation of ATP during the reaction.

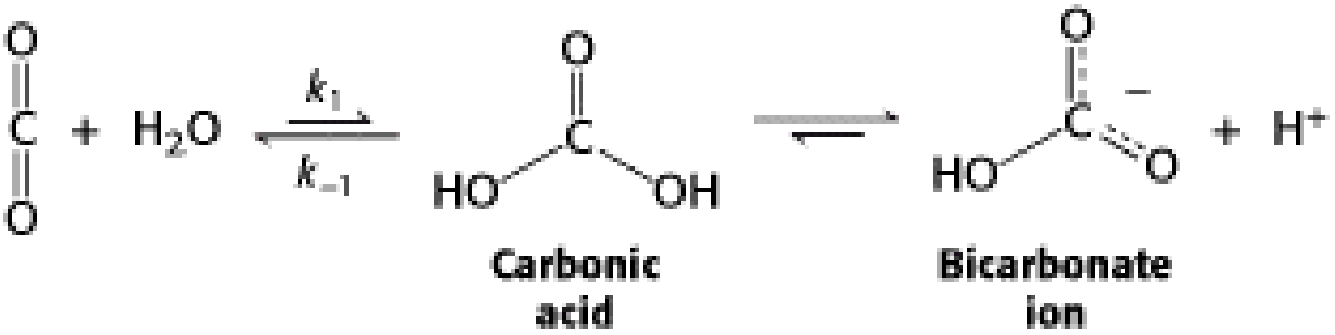
4.and they can accept electrons during oxidation-reduction reactions.

-There are certain advantages of having metals in the active site:

1) they carry positive charges so they can form relatively strong yet kinetically labile (likely to be changed) bonds.

2) They are stable in more than one oxidation state.

3) They can bind multiple ligands in their coordination sphere enabling them to



participate in binding substrates or coenzymes to enzymes.

-Mg^{+2} connects the negatively charged phosphate groups of thiamine pyrophosphate to basic amino acids in the enzyme.

-The phosphate groups of ATP are usually bound to enzymes through Mg^{+2} chelation.

- Metals can form covalent bonds with the substrates, they can form covalent bonds with the enzyme itself and they can form multiple bonds as well, not just a single bond. For example, iron which is tied to heme, can form six covalent bonds.

- that's an advantage of having metal as part of active sites of enzymes or for the purpose of having a functional protein like hemoglobin.

An example of such enzymes is **carbonic anhydrase**,

This enzyme catalyzes the formation of **carbonic acid**, and this carbonic acid can dissociate into ions, when we want to exhale CO2. In tissues there's production of

CO2 as a result of metabolism, it's converted into carbonic acid and carbonic acid dissociates into bicarbonate ion, again this reaction that takes place in tissues is catalyzed by carbonic anhydrase, this reaction is **spontaneous**, but carbonic anhydrase is still needed to speed up the reaction. (Although CO_2 hydration and HCO_3^-dehydration occur spontaneously in the absence of catalysts, almost all organisms contain *carbonic anhydrases,* because they are often coupled to rapid processes such as respiration.)

Now **why** did we bring it up in the metals section? This carbonic anhydrase requires zinc in it's active site, Zinc is found only in the +2 state in biological systems, In carbonic anhydrase, a zinc atom is bound to three imidazole rings of three histidine residues and an additional site is occupied by a water molecule.

Zinc facilitates the release of a proton from H_2O generating a hydroxide ion.

The CO_2 substrate binds to the enzyme's active site and is positioned to react with the hydroxide ion.

The hydroxide ion attacks CO_2 converting it into bicarbonate ion.

The catalytic site is regenerated with the release of the bicarbonate ion and the binding of another H_2O.

We can **have zinc just facilitating** the reaction,

Metals have different purposes, they can **do work themselves** or they can **facilitate the reaction**, for example we have a reaction in alcohol dehydrogenase in which zinc is covalently bonded to the active site, but it transfers electrons and weakens bonds and it doesn't form covalent bonds with the substrate, rather it's **positive charge pulls away electrons** making it easier for these electrons to be transferred to NAD.

What **zinc does in alcohol dehydrogenase** is the same as what **histidine does in lactate dehydrogenase**, histidine pulls of the electrons, so it binds to the protons in the substrate, making the bonds vulnerable, which is the same function as the zinc in the formerly mentioned enzyme, so it accepts protons without directly interacting with the substrate itself and thus it facilitates the transfer of these electrons to the co-substrate (NAD) .

(go back to slide 30)

Deficiency/mutations in carbonic anhydrase have been found to cause osteopetrosis (excessive formation of dense bones accompanied by anemia) and mental retardation.

PROTEIN ANALYSIS:

Biochemical techniques: are techniques that allow us to study characteristics of proteins, their solubility, their charge, their affinity to certain molecules.

To study all that about protein we:

1. **need a purified** form.

2. **need** to have a **specific way to look** at this protein.

That's all done to understand exactly what it does, to understand exactly what it looks like, so if we know it we could design a drug against this protein that can bind specifically to certain amino acids.

so it's really important to understand the state of this protein and what it looks like.

Purifying these proteins depends on **several characteristics** of these proteins: 1.solubility in solutions of certain characteristics. 2.the size of the protein. 3.the charge of these proteins. (pka of the group or isoelectric point of certain groups) 4. the affinity of binding.

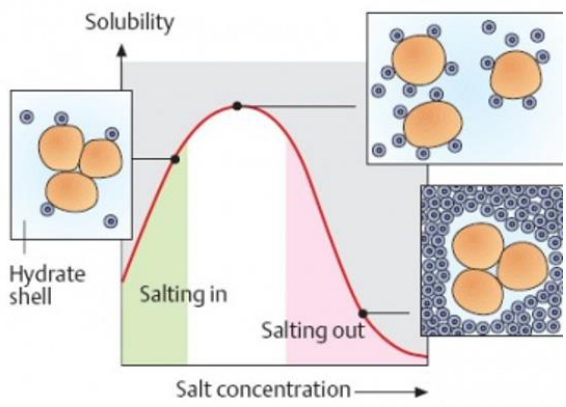
TECHNIQUES:

So now let's go over the journey of purifying and separating proteins.

Salting in … salting out

a. It depends on the solubility of proteins in a solution of certain ionic strength, certain concentration of ions (or salt) in this solution.

Explanation: if we have a protein that's added to water (no ions), we have a poor interaction between protein and water molecules, **proteins are not very soluble in pure water** → there's a preference of water molecules to interact with each other, not with the protein.



So, if we add a certain concentration of salt we'll have an interaction between the protein molecules with the salt ions, SO the protein gets **more soluble**.

If we have just **water**, we'll see **proteins preferentially interacting with each other**.

If we add salt we'll have proteins **interacting with the ions of the salt**.

Now, If we add **more** concentration of salt, proteins will again tend to interact with each other or aggregate → resulting in a large protein complex that tends to **precipitate**.

Why? what happens when we have proteins with high salt concentration is that we'll have **strong interactions between ions and water molecules**, instead of the reaction of proteins with water molecules,

Dr.ma'moun said literally explaining what happened: "the protein looks around it and it says to itself, where are ions, where are water molecules :(, I need to interact with someone- if it doesn't see water or salt ions- it says oh, I'm in a hydrophobic environment, so the hydrophobic amino acids get out" because the environment around them is no longer hydrophilic. and they **interact with each other**, SO we'll have **a protein aggregate** and eventually, the proteins precipitates.

Conclusion: reasonable amount of salt →interaction of the protein with the ions, and with the water molecules, the protein is soluble.

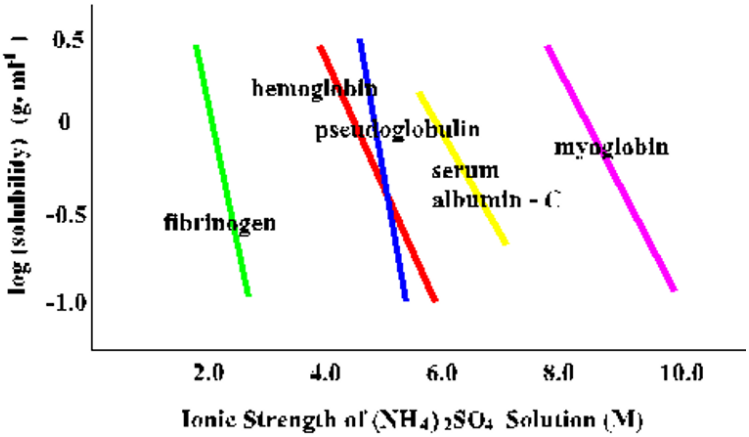
Other case → explained above.

Salting out means increasing the salt concentration so there's **no interaction** between protein and water and ions, and eventually precipitation of the protein.

-Every protein molecule has a **certain tendency** whereby it gets exposed (it's hydrophobic amino acids) in hydrophobic environment and then interacts with other molecules forming protein aggregates.

Proteins differ in their perception of **what a hydrophobic environment is**. So, proteins **will precipitate** at **different salt concentrations**, depending on the hydrophobic amino acids they contain.

Salting out can be used to fractionate proteins:



Fibrinogen → important for the formation of fibrin, it precipitate at lower concentration of salt compared to hemoglobin and albumin for example.

Hemoglobin, albumin, myoglobin precipitate on different concentrations.

Now once we have precipitation, we **take the liquid** (supernatant), **add** more **salt** and precipitate other proteins.

Every time we have a precipitation we **increase** the **salt concentration**, it depends on the proteins "thinking" this is a hydrophobic environment.

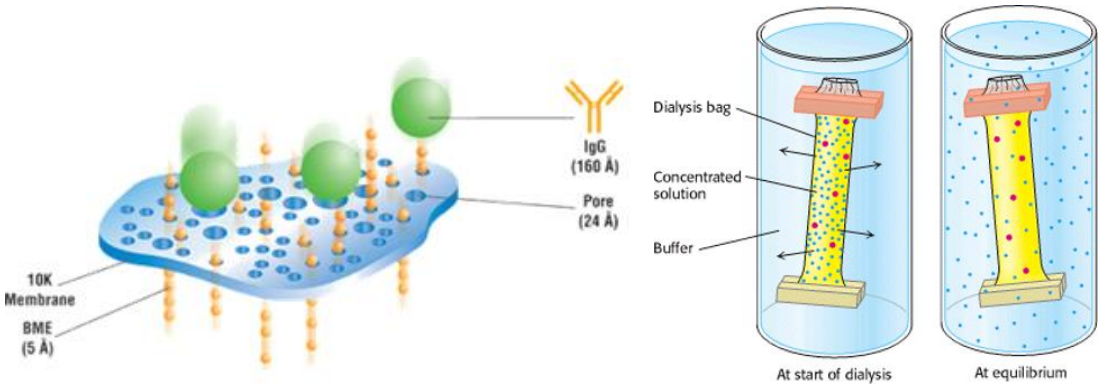
After salting out,We have a precipitate that **isn't pure** and when we take it we take with **it some salt**, but you know that in order to study the protein further we need to **remove the salt** to have the protein that's already denatured because of the salt **reform it's functional structure** meaning to form a protein with hydrophobic amino acids inside and hydrophilic amino acids outside.

Removing salt is established by another technique known as **Dialysis**.

What do we do in dialysis?

what we do is **taking** the protein **precipitate**, put it in a pored bag, these pores are small enough to prevent the proteins from passing the membrane of the bag,

but **salts** are small enough to pass → we put the bag in a certain beaker container that contains a certain solution.



What will happen is salt ions inside the bag leaving it and water molecules entering, so what we'll have is a homogenous concentration of this solution outside with what's inside the bag. here the protein will be in a low salt concentrated solution. we can take it and the protein reforms its original shape.

Dialysis: exchange of salt ions, so we would have a solution of low salt instead of the high salt concentration solution.

Disadvantage of dialysis:

1)Large number of larger proteins will still exist.

2)Smaller proteins of significance are lost.

So, **salting out** then **dialysis** what's the next step?

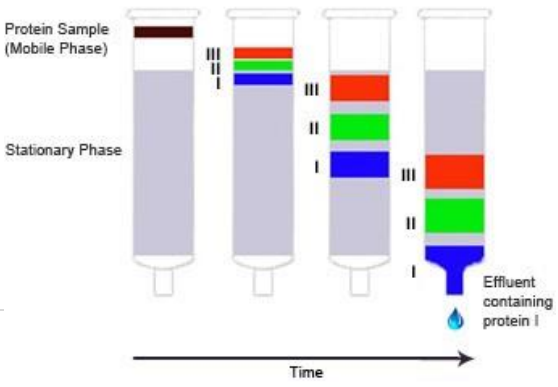
After dialysis we'll have a large number of different proteins inside the bag, like 6000 proteins that are naturally inside a cell.

(Still, we might lose some proteins, because smaller proteins might leak from the pores of the bag (small peptides), like peptide hormones, which are functional in that chain state and important)

To separate the protein/ group of proteins we want, we do something called **chromatography**, (color-graphing) .

What is chromatography?

Separation of molecules present in a liquid or gaseous environment (mobile phase) via passing



through a column (long tube) that contains an immobile phase (stationary phase).

the idea is separating different molecules that have different colors (metaphorically) by using a medium.

what we do is add a mix of proteins to medium making their separation easier, the proteins would go out of this medium one by one. In this technique **columns** are the medium.

The proteins will be separated from each other, **how?** The first molecule comes out and you collect it and then you collect the second "fraction" and so on , **each fraction is a group of proteins**.

Separation is according to certain characteristics, and depending on that we have different chromatography techniques that we can use, each for a certain characteristic.

Chromatography techniques are: Gel Filtration, Ion exchange Chromatography and Affinity Chromatograph.

Gel chromatography is also known as the **size exclusion chromatography**.

Here we have a medium known as a **stationary phase : porous beads,** it's immobile, and within it we add the sample containing the proteins known as the **mobile phase**, it moves along the immobile phase.

These proteins when separated are **separated according to size** *hence* the name: **size exclusion chromatography**.

What's the Idea? we have the stationary phase, beads packed in a column, these beads have openings, and inside them there are leading tracks from one end to the other. These openings have a ***certain size***. we **add the mobile phase** on top in a solution of the protein molecules.

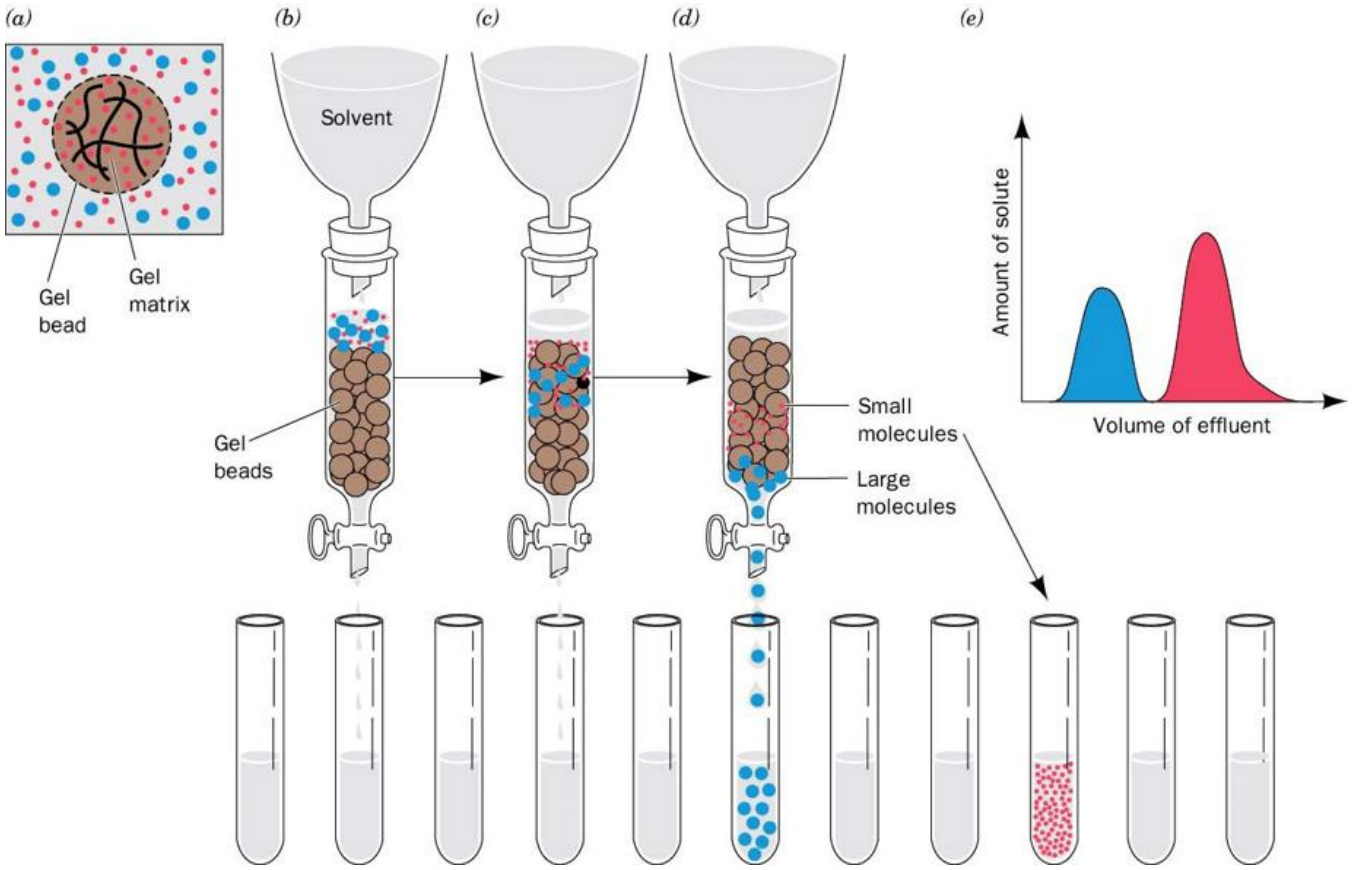
some proteins **(large)** can move **between** the beads and they **slide faster**, the **small** proteins can **enter** the beads so they go through the tracks inside the beads and it takes them *way longer time* to get out if the other end.

So, the large proteins will appear first because large proteins cannot go through the openings, so they **slide around** the beads so they move faster, so we can

collect them in fractions, multiples tubes, 1st 1 ml and then the second, and the third etc.

Eventually we'll have a tube that has the **first protein** that comes out **(the largest),** and then fractions and then a tube that contains the second protein, which is smaller. So, size separated from large to small.

You might ask, won't small proteins slide in between too? Dr.M said: there's a Higher probability of small molecules entering the pores than just sliding by.



☺Good luck