◉Sheet ○Slides

Subject:	Protein purification techniques-2
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Number:	9

In the previous lecture, we talked about different protein purification techniques. We'll continue discussing these techniques in this lecture.

Quick revision:

If you have a group of cells that have proteins, and you want to purify these proteins, you first need to homogenize the tissue samples. Then, using differential centrifugation, you separate the contents of these cells until you end up having a mixture of proteins. These purified proteins have different characteristics in terms of size, molecular weight, solubility, charge and binding affinity.

There are many techniques that depend on these different characteristics of the proteins; such as:

1. Salting in and salting out.
2. Dialysis.
3. Chromatography, and much more.

Each of these techniques depends on a certain property of the desired protein; salting in and salting out depends on the solubility of the protein. Dialysis depends on the size of the protein. Different types of chromatography depend on different properties; gel-filtration chromatography depends on the size of the proteins, ion-exchange chromatography depends on the charge/isoelectric point of the proteins, and affinity chromatography depends on the binding affinity of the desired protein.

The basics of chromatography:
Chromatography separates molecules that are found in a liquid or gaseous environment (mobile phase) by pouring it in a column (long tube) that contains an immobile/fixed material (stationary phase).

Mobile phase: The purified mixture of proteins that are going to be poured in the column.
Stationary phase: Porous beads that contain the substance which is going to bind to the desired protein(s).

Most of these techniques (ex: ion-exchange chromatography, gel-filtration chromatography, etc.…) yield various degrees of purification. However, only one technique yields only a very specific protein (the desired protein); which is the affinity chromatography.

The affinity chromatography:

It's a chromatography technique that depends on the binding affinity of the desired protein to a certain material.

Characteristics:

- The most specific separation technique.
- Gives only the desired protein.
- Depends on the binding affinity of the desired protein to the stationary phase.
- It does not contain a gel. However, it contains a resin; which itself contains a substance that has a high binding affinity to the desired protein.
- This is a very expensive technique.

Note:
For each chemical substance that is known, we can design an antibody specific to it; because every chemical is distinct than the other at a certain property, so we can design an antibody that can bind

Usually, the stationary phase contains antibodies that are very specific to the protein of interest. Then, the mobile phase gets poured inside the column, then water or a buffer is poured inside the column; to elute the solution.
Because of the antibody's specificity; no substance other than the desired protein will stay in the column.

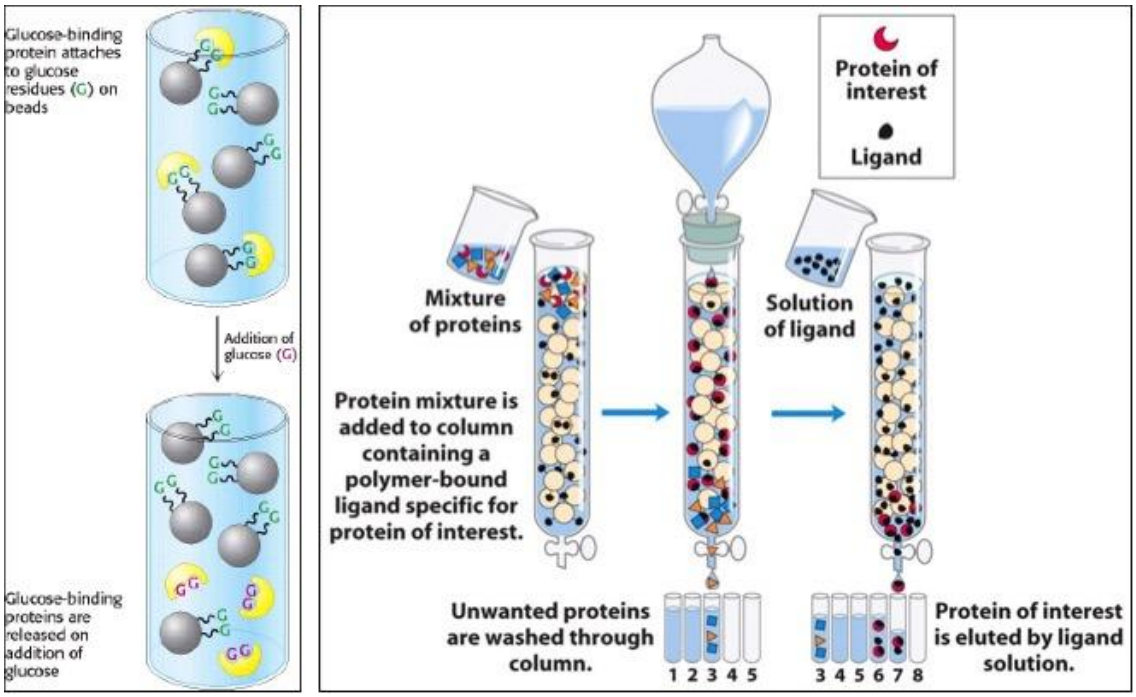
Now, to elute (extract) the desired protein, ligands that have a very specific & high binding affinity to the stationary phase must be added, these ligands will compete with the desired protein in their binding with the antibody. So eventually, the protein of interest will be separated from the antibody and gets eluted (extracted/removed). Desired protein – antibody interaction (elution) can also be disrupted by a change in pH or temperature; but this is not favorable because it may disrupt the desired protein.

Q: Why isn't salt used to elute the desired protein instead of ligands?
A: Because it doesn't make sense. Salt is used to elute the protein of interest in **the ion-exchange chromatography** because it depends on the charge of the desired protein, so adding salt (which will completely dissociate in the solution), will disrupt the electrostatic interactions by which ion-exchange chromatography is based on, and thus the salt will compete with the desired protein with it's binding to the stationary phase and eventually, the desired protein will be eluded. Adding salt in **affinity chromatography** will result in nothing because this separation method depends on the specificity between the ligand and the antibody and not on electrostatic forces.

To illustrate this technique, here is an example:
The His-Tag:
To extract a specific protein from a mixture of proteins, a high amount of this mixture must be obtained in order for the experiment to succeed, which is usually not the case when a sample of proteins is obtained. To increase the amount of the protein mixture, the gene that codes for the desired protein is extracted, and then the gene is modified by adding a DNA sequence that will code for the production of 6 to 10 histidine residues at the N or C terminals of the desired protein. Then, this modified gene gets injected in bacterial cells which will produce the exact same desired protein with an extra 6 to 10 histidine residues at one of its' ends. These histidine residues are called the histidine tag, or His-Tag.

And since bacterial cells have a high proliferation rate, the number of bacterial cells will increase rapidly and therefore, the production of the desired protein with the His-Tag will increase to a point where the experiment can be held successfully. Then these bacterial cells will undergo homogenization and centrifugation until the protein mixture is obtained. This protein mixture will undergo affinity chromatography where Nickel is attached to the stationary phase, which binds specifically to the imidazole rings of the His-Tag. And to extract the desired protein, a high concentration of imidazole can be added (ligand) which will compete for the nickel that is attached to the His-Tag's imidazole side chains, and the protein will get eluted, because the ligand-antibody interactions are based on non-covalent interactions, so increasing the concentration of the ligand will result in the protein's elution.

Another example is the plant protein "Concanavalin A", which binds to glucose with high affinity, and can be purified by passing a protein mixture through a column of beads attached to glucose residues. Concanavalin A, but not other proteins, binds to the beads. The bound Concanavalin A can then be released by adding a concentrated solution of glucose.



Gel electrophoresis:

It's the movement of a charged particle through a gel under the influence of an electric field.

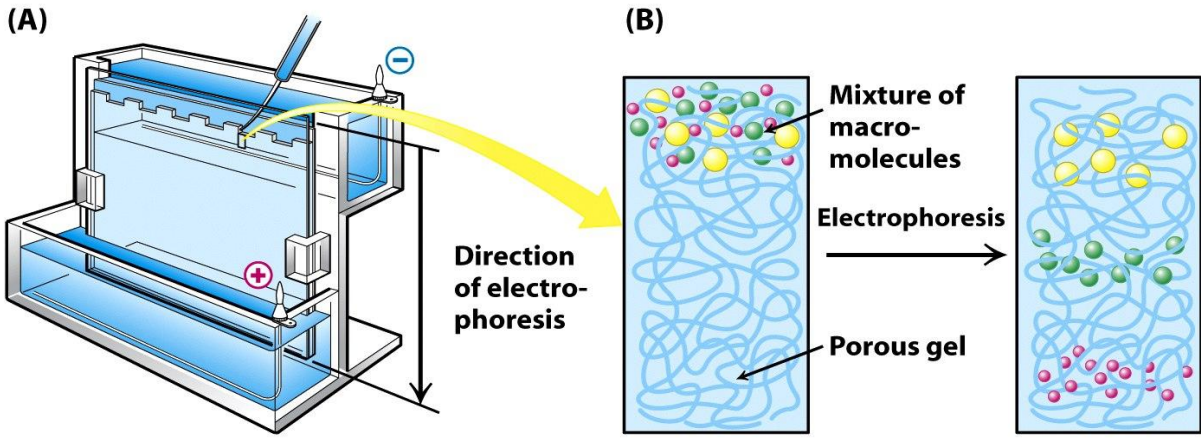
- This technique requires the presence of gel and an electric field; as the name implies.
- The gel must be porous; so that the substances can move through these pores.
- The gel was initially a liquid material, and then it got solidified.

<u>Gel preparation:</u>
There are some types of gels that are prepared by cooling, such as the agarose gel.

<u>Agarose gel preparation:</u>

- Agarose is mixed and then boiled in the microwave for a certain amount of time.
- Once it gets out of the microwave, it must be poured in a plate until it solidifies.

There are other types of gels that are called chemically induced gels; which are prepared in the laboratory using chemical reactions to produce the jelly material. An example of this type is the polyacrylamide gel.



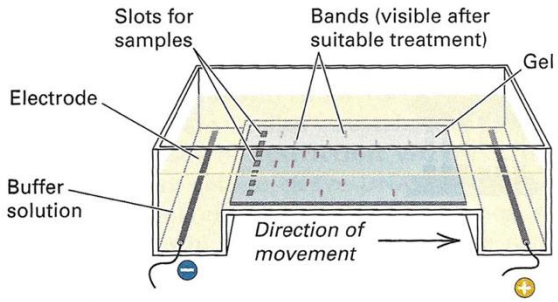
Mechanism:

- When the gel is still in it's liquid state, a comb is placed in the beginning of the gel, and wait until it solidifies.
- When the gel solidifies, the comb is removed, which leaves spaces (wells) inside the gel.
- The protein mixture is poured inside these spaces.
- The gel and the inserted protein mixture are then put within a watery or a buffer solution, and then it will be subjected to an electric field.

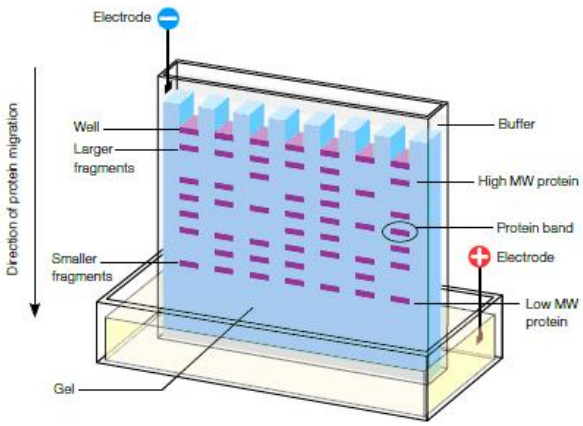
The electric current will move from the negative to the positive electrode. Induced by the electric field, the negatively charged proteins will now move across the gel, and this depends on several factors such as:

- The net charge of the protein.
- The size/molecular weight of the protein (Number of amino acids affects the molecular weight).
- The shape of the protein.

Agarose gel electrophoresis of DNA



← Agarose gel electrophoresis (DNA)



← Polyacrylamide gel electrophoresis (Proteins)

The differences between the agarose and the polyacrylamide gel electrophoresis:

	Agarose gel	**Polyacrylamide gel**
Usage	Commonly used for DNA and RNA electrophoresis.	Commonly used for protein electrophoresis.
Shape of pores	The pores that this type of gel forms upon solidifying are not in a uniform shape.	The pores that this type of gel forms upon solidifying are in a uniform shape.
Resistance and proteins separation	It does not provide a high resistance for the movement of proteins in it. Which means that it doesn't differentiate between proteins that have close molecular weights.	It provides a high resistance for the movement of proteins in it. Which means that it can separate proteins even when they have very close molecular weights.
Direction of the gel	It's technically a horizontal gel electrophoresis, because when prepared, the liquid gel will be poured on a horizontal plate. And when the electric field is applied, the substances will move horizontally across the jelly plate.	It's technically a vertical gel electrophoresis, because when prepared, the polyacrylamide gel will be placed between 2 vertical slides. And when the electric field is applied, the substances will move vertically across the gel that's between the slides.

Polyacrylamide gel electrophoresis (PAGE):

Polyacrylamide gel electrophoresis is divided into two main techniques:
I) Native Polyacrylamide gel electrophoresis (Native PAGE).
II) Sodium Dodecyl Sulfate Polyacrylamide gel electrophoresis (SDS-PAGE).

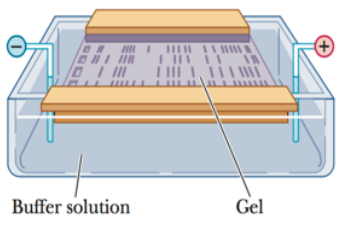
I) Native Polyacrylamide gel electrophoresis (Native PAGE):

In "Native PAGE", the protein is used in its native (original, folded) state. Thus, movement of proteins in the gel doesn't depend on size specifically, they move through the gel according to their molecular weight, size, shape and charge.

Samples are placed in wells (produced by the comb) near the negative electrode (Fig 5.10), an electric current is applied, proteins start moving, and we can predict the results depending on:

A) **Molecular weight**: Samples with lower MW tend to move faster than those with higher molecular weights.
B) **Size**: Samples with smaller size will move faster than those with larger sizes.
C) **Charge**: The more negatively charged the protein is, the faster it moves toward the positive electrode.

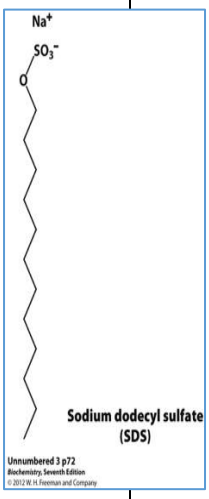
NOTE: This method gives us a very small prediction about the MW of the protein, it doesn't give an accurate prediction about the MW, because there are other factors (shape & charge) involved in this method.
To make an exact prediction about the MW of the protein, we have to fix the other two variables and remove their effect. This is done by **"SDS-PAGE"**.



■ **FIGURE 5.10 The experimental setup for gel electrophoresis.** The samples are placed on the left side of the gel. When the current is applied, the negatively charged molecules migrate toward the positive electrode.

II) Sodium Dodecyl Sulfate Polyacrylamide gel electrophoresis (SDS-PAGE).

This technique utilizes **a negatively charged detergent (sodium dodecyl sulfate)** to <u>denature</u> and solubilize proteins. Thus, proteins move according to their molecular weight only.



A) Denaturing the protein (Using SDS for example) will remove the shape's effect. SDS won't break the disulfide bridges, so we have to break them down by using reducing agents such as β-mercaptoethanol (βME) and dithiothreitol (DTT).
So we will have the primary structure only.

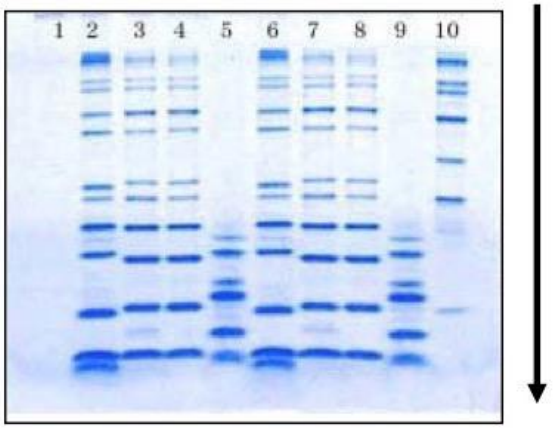
B) To remove the charge's effect, SDS (negatively charged) binds to all of the <u>polar amino acids</u>. It makes proteins have a <u>uniform negative charge.</u>

All of the proteins are now denatured and heavily loaded with negative charges. They have a uniform shape and charge, so they will move in the gel <u>only according to their molecular weight.</u>

What can we learn from SDS-PAGE?

A- Whether the sample has proteins or not.

Where there is a band, there is a protein. Absence of bands means the absence of proteins. Sample #1 has no proteins because it has no bands.



B- The sizes of the proteins.

Low molecular weight proteins or polypeptides move further away than high molecular weight proteins. We can tell that sample 9 for example has proteins with low molecular weight.

C- We can tell if a certain protein exists in a certain sample.

For example: There are proteins in sample #5 that don't exist in sample #2, due to the difference in their profiles.
Sample #2 has the same proteins that exist in sample #6.

Note: The proteins in the samples are denatured, so they are now polypeptides. Its better to say that each band represents a polypeptide not a protein.

CHECK SLIDES 31-34, PRESENTATION 5 FOR DR. MA'MOON FOR SOME IMPORTANT QUESTIONS.

Isoelectric focusing:

This method depends on the isoelectric points of the proteins. An isoelectric point is the pH at which the protein carries no net charge.

We use a gel prepared with a pH gradient. As proteins migrate through the gel, they encounter regions of different pH, so the charge on the protein changes. Eventually, each protein reaches the point at which it has no net charge—its isoelectric point—and no longer migrates (it won't be affected by the -ve & +ve electrodes). Each protein remains at the position on the gel corresponding to its isoelectric point(pI), allowing for separation of proteins.

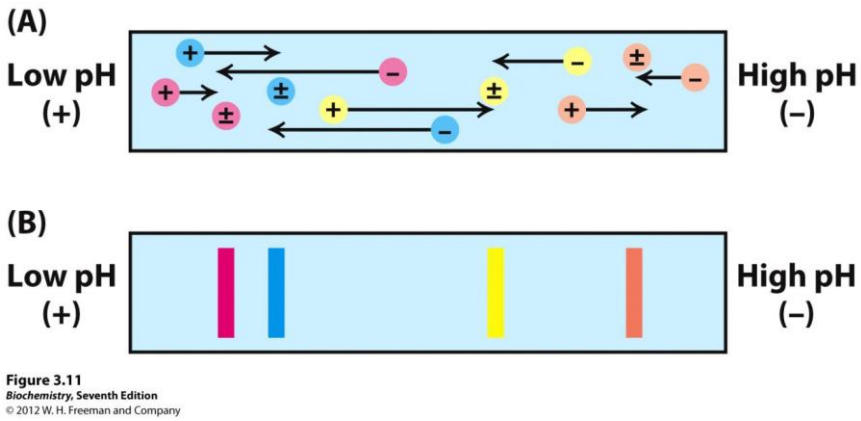


Figure 3.11
Biochemistry, Seventh Edition

Two-dimensional gel electrophoresis (2D-PAGE):

In 2D-PAGE, proteins are separated by isoelectric focusing first, then through an SDS-PAGE.

We separate the proteins according to their isoelectric points in an isoelectric focusing gel, then we load them with SDS to denature them and give the proteins a uniform negative charge. After that, we apply an electric current, and the proteins will move under the effect of their molecular weights.

Since some proteins might have the same isoelectric point or the same molecular weight, this technique allows protein separation based on both charge and size at the same time.

To understand this method better, click here.

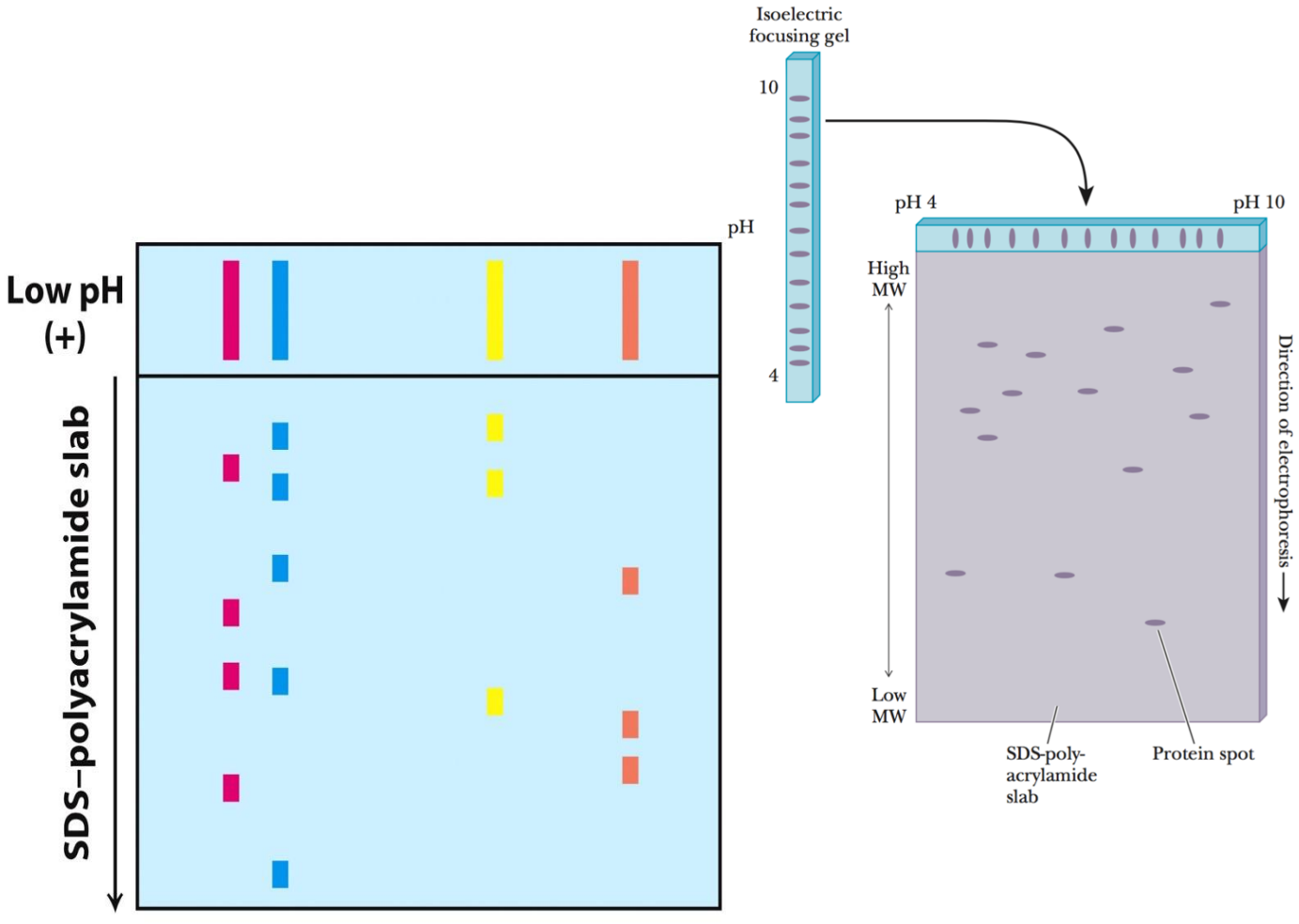


Figure 3.12a
Biochemistry, Seventh Edition

Immunoassays:

A lot of techniques in biochemistry depend on immunology (antigen and antibody binding) to detect proteins and their characteristics. One of these techniques is called "ELISA" technique.

ELISA (Enzyme-linked immunosorbent assay):

Enzyme-linked: The process involves the action of enzymes
Immuno: It involves Antigen/Antibody binding.
Sorbent: Adsorption of a certain antibody or antigen to the plate.

- This method can detect the protein with a level of less than a nanogram. It is very specific (depends on antigen/antibody binding).
- This method is used to detect many viruses and diseases like Hepatitis, HIV, etc.

Clinical application: Pregnancy test.

When the egg is fertilized by the sperm, a zygote forms. This zygote starts producing the "Human chronic gonadotropin (HCG)". This hormone is used as an antigen in this test. The common sample used in pregnancy tests is a urine sample.

Procedure:

-We bring a sorbent plate (Plastic, polystyrene) that has the ability to adsorb an antibody to it. The antibody is specific for the antigen we are using (HCG in this example).
-Once the antibody is attached to the plate, we pour the urine sample on the plate.
-If the hormone is present, it will bind to its antibody. If not, nothing will bind to the antibody.
-After that, we add another antibody that can bind to the already existing "antigen-antibody" complex. This second antibody is linked

to an enzyme that will only be activated if the antibody binds to the "antigen-antibody" complex.
-If its activated, it will turn a colorless solution (added later as a substrate for the enzyme) into a colored one, and we will get a positive test. Otherwise, the solution will remain colorless, and we'll get a negative test. The intensity of the color indicates how much antigen there is in the sample.
Note: If the egg wasn't fertilized, the HCG hormone won't be produced, and we will get a negative test.
To understand the general ELISA method better, click here.