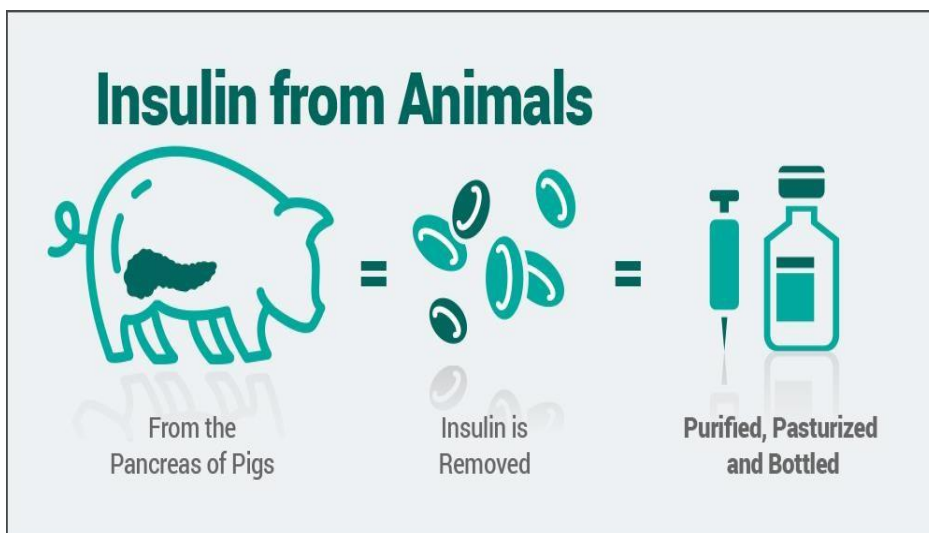


Course handout

Production of biomolecules in animal cell systems



Designed for first-year Master's students
Option: Biotechnology and molecular pathology
Presented by Dr. Selma HOUCHE

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➤ **Information about the course**

1. Necessary information

Faculty: Natural and life sciences

Department: Biochemistry

Title of the Master: Biotechnology and Molecular Pathology

Target audience: 1st year Master

Title of the Teaching Unit: Fundamental Unit

Subject title: Production of biomolecules in animal cell systems

Credits: 6

Coefficients: 3

Semi-annual hourly volume: 67.5

Schedule: Monday: 8:00 a.m. - 9:30 p.m.

Room: Amphi 4

Teacher: Dr. Selma HOUCHI

Contact: email houchi.selma@univ-setif.dz

Availability: Monday, Tuesday, Wednesday from 8:00 a.m. - 12:30 p.m., Office A13.

Answer on the forum (Moodle platform): any question relating to the course must be posted on the dedicated forum, so that you can all benefit from my answer, I undertake to answer the questions posted within 48 hours.

By email: I undertake to respond by email within 48 hours of receipt of the message, except in the event of unforeseen circumstances, I draw your attention to the fact that the preferred communication channel is the forum, the email is reserved for " emergencies" (in the event of a problem accessing the platform) and it must be used with discernment.

2. Presentation the course

The production and extraction of biomolecules from animal cells are crucial processes in biotechnology, biochemistry, and medicine. These biomolecules, including proteins, enzymes, antibodies, and hormones, are essential for various applications ranging from fundamental research to drug development. The world of biomolecules is a complex riddle! The pieces are numerous, some are well-known, others less so... The course on Production of biomolecules in animal cell systems provides a detailed understanding of the methods used in the production and extraction of valuable molecules such as insulin, interferons, and erythropoietin, as well as their structure, biosynthesis, role, and regulation. Studying this course involves being able to identify the mechanism behind a wide range of human diseases. Comprehending better has not only revolutionized medicine and biotechnology, but has also paved the way for new possibilities in the treatment of various diseases.

3. Content

According to the outline of the academic Master's training offer in biotechnology, specialty biotechnology and molecular pathology, the course Production of Biomolecules in animal cell systems contains three chapters whose main objective course is to provide students with an in-depth understanding of biomolecules in animal cell systems such as insulin, interferons and erythropoietin and other biomolecules in the tutorials.

This course is structured into three main chapters, each aimed at achieving specific objectives to enable students to master key concepts and essential techniques.

Introduction: Biomolecules in animal cellular systems

1. Insulin

- a. Structure and biosynthesis
- b. Physiological role
- c. Regulation
- d. Production

2. Interferon

- a. Structure and biosynthesis
- b. Physiological role
- c. Regulation
- d. Production

3. Erythropoietin

- a. Structure and biosynthesis
- b. Physiological role
- c. Regulation
- d. Production

By following this course structured into three chapters, as well as the biomolecules covered in the tutorials, students will develop a comprehensive understanding of biomolecules in animal cell systems, from their biosynthesis to their extraction and purification. Each chapter is designed to strengthen the theoretical and practical skills required to flourish in biotechnology and biomedical sciences.

4. Pre-requisites

In order to optimize the comprehension and success of this course, it is recommended that students possess prior knowledge and skills in Biochemistry, analysis techniques, Genetics, Cell Biology, and a certain level of knowledge in Biotechnology.

Verify that you possess the requisite knowledge to participate in this course in the best possible manner by clicking on this link

<https://snv-cours.univ-setif.dz/cours/index.php?id=591>

Please use the provided username and password to log in, then select the "my courses" section and choose the course "Production of Biomolecules in Animal Cellular Systems".

You can take the test as early as the first week, and you can do it as many times as you need to during that time. If the grade you get isn't good enough, you'll be sent to an auto-formation course that you can do at your own pace and is available on the same platform for online learning by using the same link.

5. Learning objectives

The course on Production of Biomolecules in Animal Cellular Systems aims to provide students with a comprehensive and practical understanding of biomolecules, as well as the traditional methodologies used to produce and extract biomolecules from animal cells.

Acquiring knowledge about the characteristics of biomolecules and the ability to produce and extract molecules from animal cells is highly significant in the fields of biotechnology and biochemistry.

This course assists students in acquiring the knowledge and skills necessary to succeed in this evolving field. By integrating traditional approaches with recent biotechnology innovations that are being studied in the course of production of biomolecules in eukaryotic and prokaryotic cells, students will be prepared to confront future challenges and make a significant contribution to the field of biotechnology.

***CHAPTER 1. Introduction to
Biomolecules in Animal Cell
System***

I. Introduction to Biomolecules in Animal Cell Systems

Biomolecules are compounds synthesized by living organisms that play a key role in biological, industrial, and medical processes. In animal cellular systems, these biomolecules mostly consist of proteins, carbohydrates, lipids and nucleic acid. The production and extraction of these biomolecules are crucial for scientific research, drug development, and biotechnological applications. Understanding the structure, biosynthesis, function, and generation of these biomolecules is essential for several applications in biotechnology, medicine, and scientific research. This chapter aims to provide a comprehensive understanding of the characteristics and methods used to produce biomolecules from animal cells.

It establishes the necessary foundations for understanding biomolecules in animal cellular systems. By mastering these ideas, students will be better equipped to approach the processes of production of biomolecules, and to understand their functioning, interaction, and significance in biotechnological and medical applications. A thorough understanding of these principles is crucial for any professional in the field of life sciences.

I.1. Protein

The term "Protein" originates from the Greek language, denoting its literal meaning as "of utmost significance" or "holding the highest rank". These components are crucial for the optimal functioning of living cells, both in terms of their abundance (constituting over 50% of the cell's dry weight) and their quality (serving as structural proteins, enzymes, and defence mechanisms). Proteins consist of carbon, hydrogen, oxygen, nitrogen, and many of them also include Sulphur (S) and occasionally phosphorus.

Proteins are complex macromolecules that are created through the condensation of many amino acids. They can be categorised into two separate groups:

Proteins made entirely of amino acids are known as holoproteins. Heteroproteins, also known as conjugated proteins, are distinguished not only by the amino acid sequence but also by the presence of a prosthetic grouping of different kinds.

I.1.1. Structure

Four levels of structural organisation can be used to describe proteins. A polypeptidic chain consisting of a linear sequence of amino acids defines the primary structure of a protein. This chain, which is arranged in the shape of a beaded string of "pearls" of amino acids, makes up the protein molecule's backbone. As a result, this spiral underwent twists and reversals to reach more sophisticated molecular organisation levels, such as secondary, tertiary, and quaternary structures.

I.1.1.1. Amino acids

The molecules in question are defined by the existence of a group consisting of a carboxylic

acid function (COOH) and an amine function (NH₂), as their name suggests. There are a total of twenty essential amino acids, each possessing two functional groups: an amine group (—NH₂) and a carboxylic acid group (—COOH). Therefore, an amino acid has the ability to function as both a base, which accepts protons, and an acid, which donates protons. All amino acids share similarities except for their unique R group, which is situated in the third position. Every amino acid demonstrates distinct chemical properties, along with a varying level of acidity or alkalinity, determined by the arrangement of atoms in its R group (**Figure 1**).

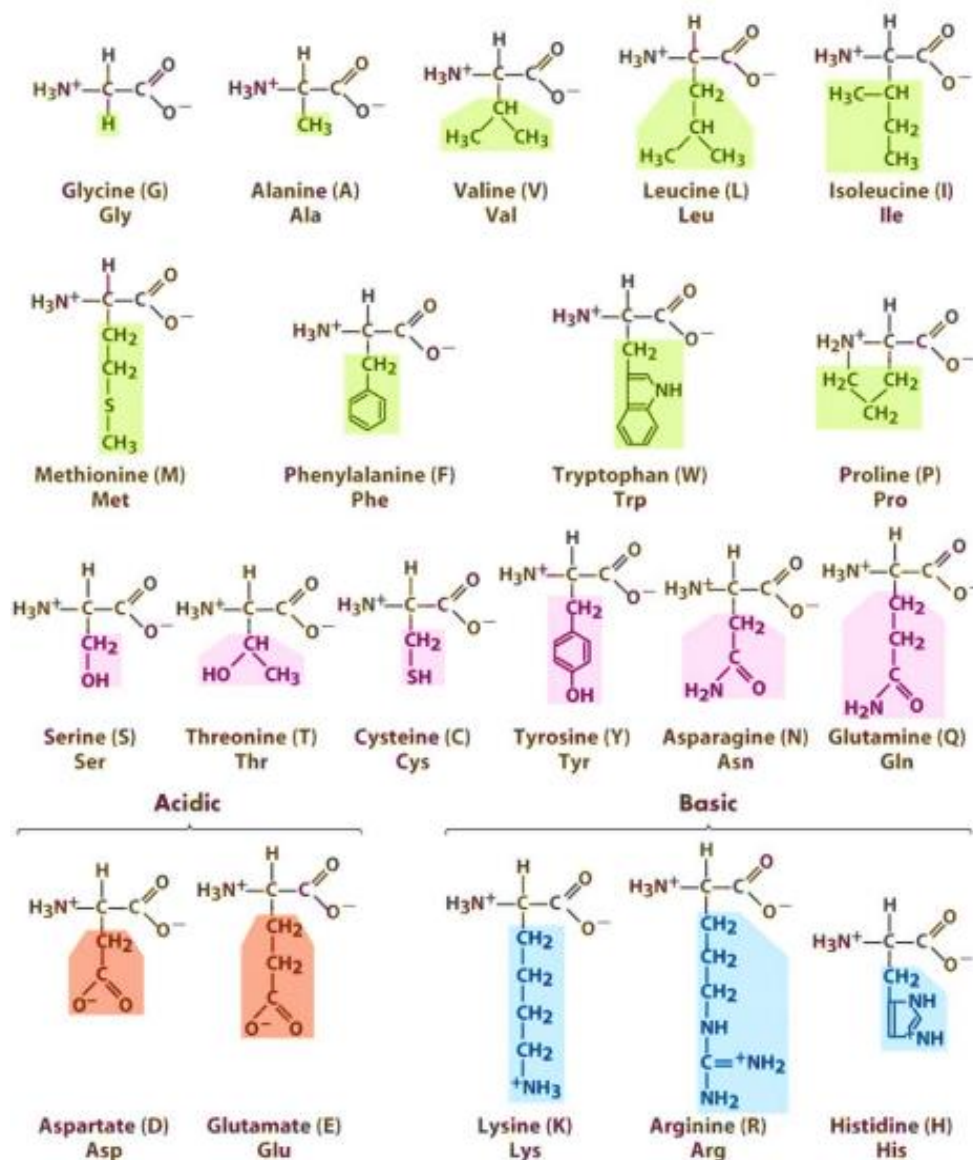


Figure 1. Structure of the 20 α -amino acids

I.1.1.2. Primary structure

The primary structure of a protein is defined as the sequence of amino acids linked together to form a polypeptide chain. Each amino acid is linked to the next through peptide bonds created during the process of protein biosynthesis (**Figure 2**). The kind and sequence of the amino acids

that make up each protein determine its unique properties. The 20 amino acids can be compared to an “alphabet” of 20 letters, used to form “words” (proteins). In the same way that it is possible to modify the meaning of a word by substituting one letter for another (Hard $\rightarrow$ Card), it is possible to generate a new protein with a different function by replacing an amino acid or by modifying his position. Sometimes the new sequence has no meaning (Hard $\rightarrow$ Hkrd), just as sometimes alterations in the combination of amino acids lead to non functional proteins.

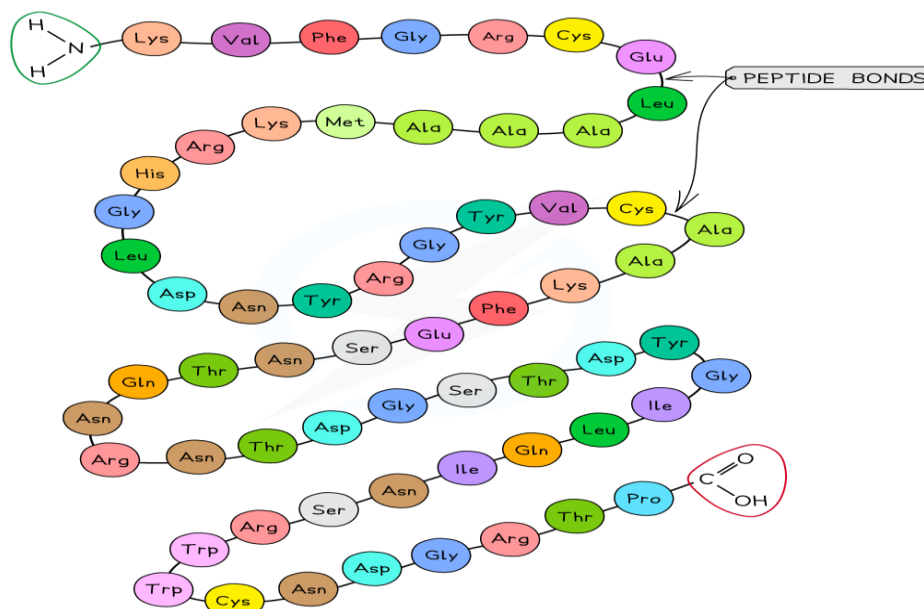


Figure 2. Primary structure of proteins

I.1.1.3. Three-dimensional structure of proteins

a Secondary structure

Proteins do not occur as linear chains of amino acids, but undergo twists and responses on themselves, forming their secondary structure. The alpha (α) helix is the most common secondary structure. In this structure, the primary chain coils around itself and is stabilized by hydrogen bonds between the NH and CO groups, approximately every four amino acids.

The beta (β) pleated sheet is a distinct secondary structure in which primary polypeptide chains associate side by side through hydrogen bonds to form a folding ladder-like structure, without coiling. In this context, hydrogen bonds can stabilize distinct regions of the same chain reacted on itself in an accordion manner, or even connect different polypeptide chains. Unlike alpha helices, where hydrogen bonds lie only on distinct parts of a single chain, it is possible for a polypeptide chain to have both alpha helices and beta pleated sheets (**Figure 3**).

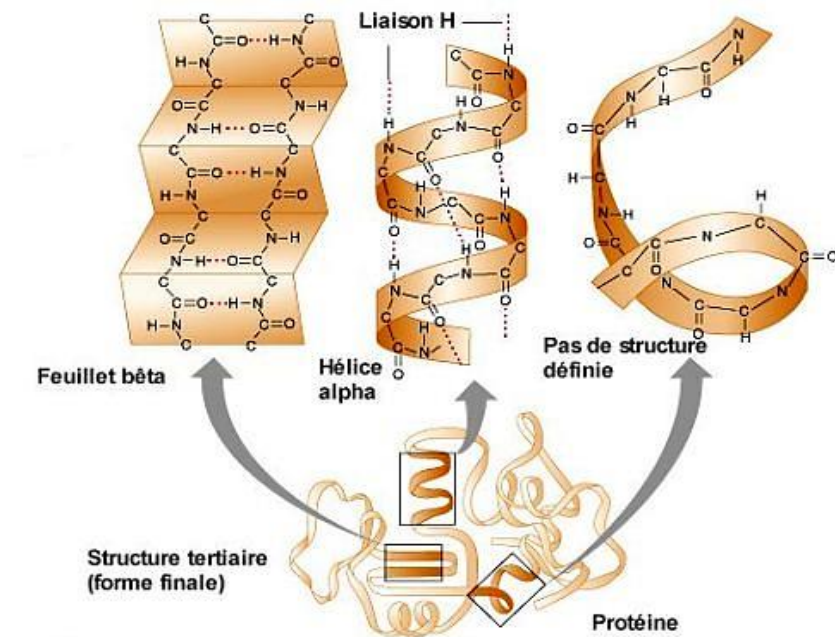


Figure 3. Levels of structural organization of proteins

b Tertiary and quaternary structure

A large number of proteins achieve their tertiary structure, which is a very specific configuration resulting from secondary structure. In this structure, helical or folded segments of the polypeptide chain arrange relative to each other to form a globular molecule. The interactions maintaining the tertiary structure involve bonds (covalent, hydrogen, etc.) between often distant amino acids on the primary chain (**Figure 3**).

The quaternary structure refers to the specific arrangement of multiple peptide chains forming a single higher entity which is the only one capable of fully ensuring biological functions. Hemoglobin, as illustrated in Figure 4, exhibits a level of structural organization where two α chains are linked to two β chains (**Figure 4**).

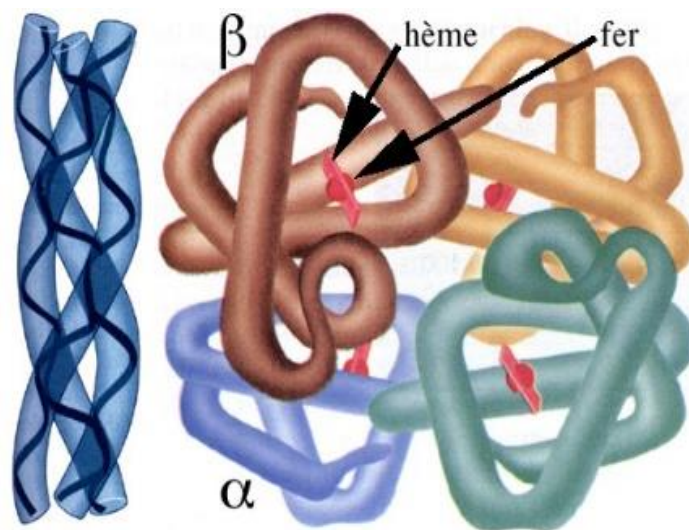


Figure 4. Quaternary structure of Hemoglobin

I.1.2. Function

The three-dimensional conformation of the protein determines its unique characteristics and influences its biological function. Proteins are generally classified into two categories based on their overall structure: fibrous proteins and globular proteins.

I.1.2.1. Fibrous proteins

Fibrous proteins are referred to as structural proteins because of their dominant role in construction in vertebrates. They are characterized by their linear structure, their insolubility in water and their high stability, thus providing mechanical support to tissues and tensile strength. The most important fibrous proteins include collagen, keratin, fibrinogen and muscle proteins.

I.1.2.2. Globular proteins

Unlike fibrous proteins, globular proteins are characterized by their spherical shape and high solubility, and they occupy an essential place in metabolism. Globular proteins include albumins, globulins, casein and protein hormones, which are present in abundance in animal cells, blood serum, milk and eggs. The main functions of globular proteins in humans will be discussed below.

a Catalysis process

Enzymes play an essential role in virtually every biochemical reaction in the body, increasing the speed of chemical reactions by at least a million times. For example, salivary amylase found in saliva catalyzes the breakdown of starches, while oxidases facilitate the oxidation of food fuels.

b Transportation

Hemoglobin has the function of carrying oxygen through the bloodstream, while lipoproteins are responsible for transporting lipids and cholesterol. In addition, the blood contains other transport proteins dedicated to transporting iron, steroid hormones and other substances.

c pH control

Many plasma proteins, such as albumin, have the ability to act as acids or bases within a buffer system. Their role is to regulate fluctuations in blood pH by absorbing or releasing H^+ ions.

d Regulation of metabolism

It is a complex process that involves a series of biochemical mechanisms aimed at maintaining the balance of metabolic reactions in the body.

Polypeptide hormones and protein hormones play essential roles in the regulation of metabolic activity, growth and development. For example, growth hormone is an anabolic hormone essential for optimal growth, while insulin helps regulate blood sugar.

e Protection of the body

Antibodies, highly specialized proteins, have the function of recognizing and neutralizing bacteria, toxins and certain viruses. They play an essential role in the immune response by

protecting the body against pathogens and foreign substances. Furthermore, complement proteins, present in the blood circulation, strengthen the effectiveness of the immune system and promote the activation of the inflammatory reaction, a non-specific defense mechanism of the body.

I.2. Lipids

Lipids are biological molecules essential for survival, having diverse and essential functions in cellular constitution, energy metabolism, the transmission of biochemical signals, as well as the protection of organisms.

These are esters formed from alcohol and fatty acids, characterized mainly by their insolubility in water. However, they can be soluble in organic solvents such as acetone, alcohol, chloroform and ether. There are two categories of lipids:

Fatty acids are also known as simple and complexes which are lipids composed of various lipids such as glycerolipids, sphingolipids, ceramides and cholesterol.

I.2.1. Classification

I.2.1.1. Fatty acids

Fatty acids can be classified in various ways based on their structure. Based on the length of the carbon chain, which can vary from 4 to more than 24 carbon atoms. Based on their level of unsaturation, which corresponds to the number of carbon-carbon double bonds present in the molecule, we can distinguish:

- Saturated fatty acids.
- Monounsaturated fatty acids are characterized by the presence of a double bond.
- Polyunsaturated fatty acids are characterized by the presence of several double bonds.

I.2.1.2. Glycerolipids

It is possible to categorize lipids into simple glycerolipids, also called glycerides, and complex glycerolipids, known as phosphoglycerolipids. While glycerides are primarily involved in energy storage, phosphoglycerolipids help build cell membranes and egg yolk.

a Glycerides

They are mainly composed of triglycerides, also known as neutral lipids. They are formed by esterification reactions of glycerol with three fatty acid molecules. An example of a homogeneous triglyceride is one where all three fatty acids are identical (**Figure 5**). Mixed triglycerides are the most common due to the diversity of fatty acids they contain.

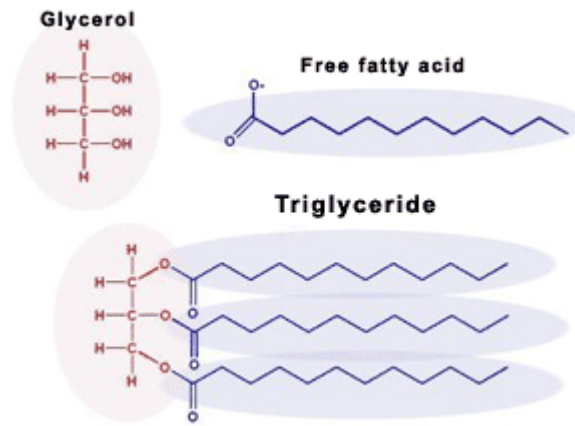


Figure 5. Structure of homogeneous triglyceride

b Phosphoglycerolipids

Lecithins, also known as phosphatidylcholines, are found in sources such as egg yolk, soy, as well as various tissues such as the pancreas, liver, and nervous tissues. Their composition includes fatty acids, glycerol, phosphoric acid and nitrogen alcohol (**Figure 6**).

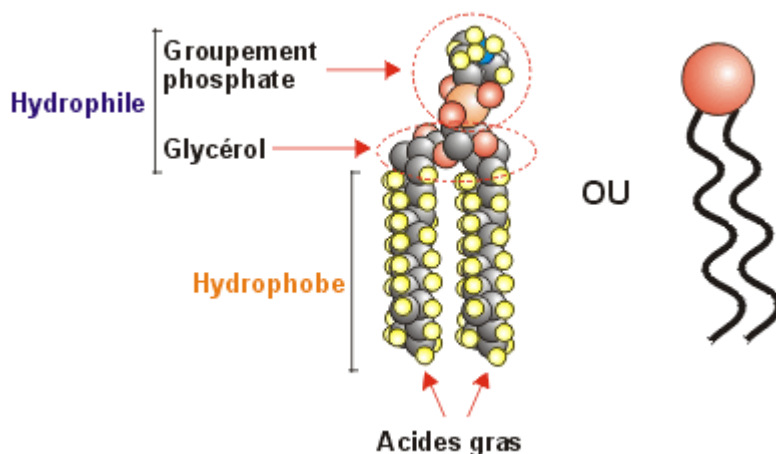


Figure 6. Structure of Phosphoglycerolipide

I.2.2. Function

I.2.2.1. Energy production

Triglycerides represent the main source of stored energy in the human body. They are stored in adipocytes and can be metabolized to produce energy in the form of ATP.

I.2.2.2. Components of cell membranes

The plasma membrane which envelops cells is found both in unicellular beings and in multicellular, whether prokaryotes or eukaryotes. Its essential role is to separate the cellular interior from the exterior. Phospholipids constitute the double lipid layer of cell membranes, thus creating a semi-permeable barrier. Cholesterol stabilizes cell membranes and regulates their fluidity.

I.2.2.3. Cellular Signaling

Lipid signaling encompasses all biochemical mechanisms of cellular communication involving lipids that interact with a target protein, such as a receptor, a kinase or a phosphatase. This interaction results in the modulation of other cellular processes in response to lipid mediators.

Steroid hormones regulate a variety of physiological processes, such as cortisol, testosterone, and estrogen. Prostaglandins, derived from fatty acids, act as mediators of inflammation and pain.

I.3. Carbohydrates

Carbohydrates represent approximately 1 to 2% of cell mass. They are composed of carbon, hydrogen and oxygen. These last two elements are present in a ratio of 2:1, similar to that of water, hence the name used is carbohydrates. They are organic compounds that are distinguished by the presence of carbon chains containing hydroxyl groups, as well as aldehyde or ketone functions. They are classified according to their size and their solubility into monosaccharides, disaccharides and polysaccharides. Monosaccharides serve as the fundamental units for the formation of other types of carbohydrates. Generally, the solubility of a carbohydrate molecule in water decreases as its size increases.

I.3.1. Classification

I.3.1.1. Monosaccharides

Monosaccharides, also called simple sugars, consist of a single chain, linear or cyclic, composed of 3 to 6 carbon atoms. Their general formula is $(CH_2O)_n$, where n represents the number of carbon atoms. The most common are those that contain 5 or 6 carbon atoms, and are called respectively pentoses ($n = 5$, e.g. ribose and deoxyribose) or hexoses ($n = 6$, e.g. glucose, fructose and galactose).

I.3.1.2. Disaccharides

A disaccharide is formed by the condensation of two monosaccharides during a synthesis reaction. The two molecules are connected by an osidic bond or a glycosidic bond, formed by the bonding of two hydroxyl groups with the loss of a water molecule. The main sugars, having the formula $C_{12}H_{22}O_{11}$, are sucrose, maltose and lactose.

I.3.1.3. Polysaccharides

Polysaccharides are polymers of glucose, such as starch, glycogen and cellulose. They are a category of polymeric carbohydrates characterized by their complex structure, thus distinguishing them from monosaccharides such as glucose and fructose, as well as disaccharides such as sucrose and lactose. They are also known as glycans, polysaccharides, polysaccharides or complex carbohydrates.

I.3.2. Function

I.3.2.1. Energy production

One of the main functions of carbohydrates is to serve as a readily available source of energy, essential for cellular metabolism, particularly in muscle, brain, heart and blood tissues. Glucose provides the primary energy substrate for the process of cellular respiration, resulting in the production of adenosine triphosphate (ATP), while glycogen represents a short-term energy reserve in animals that can be mobilized quickly.

I.3.2.2. Structural Role

Certain carbohydrates play essential structural roles in living organisms. For example, plants synthesize cellulose and hemicellulose fibers, while animals produce chitin to form their shell or their claws. Likewise, bacteria use polysaccharides to provide the structure of their membrane. Furthermore, glycosaminoglycans are crucial components of connective tissues, contributing to their mechanical properties.

I.3.2.3. Cellular Signaling

Carbohydrates may play a crucial role as a means of cellular communication. The oligosaccharides present in the glycosylations of glycoproteins and glycolipids present a great diversity in terms of composition, length, branching and types of linkages between the oses. This diversity offers the possibility of precise recognition, thus promoting interactions and cellular communication with the environment, particularly in the case of membrane proteins and lipids, as well as in the targeting and action at a distance of secreted proteins.

I.4. Nucleic acids

Nucleic acids represent fundamental biomolecules that play a crucial role as carriers and vectors of genetic information within cells. They mainly fall into two categories: deoxyribonucleic acid (DNA) and ribonucleic acid (RNA).

I.4.1. Types

I.4.1.1. Deoxyribonucleic acid (DNA)

a Structure

The double helix consists of two antiparallel strands of DNA that wrap around a central axis. The nitrogen bases of the strands are paired by specific hydrogen bonds: adenine with thymine, and cytosine with guanine.

b Function

DNA, like a precious librarian, meticulously preserves genetic secrets within its chromosomes. In eukaryotic cells, it is found not only in the nucleus, but also in the mitochondria and, exclusively in plants, in the chloroplast.

I.4.1.2. Ribonucleic acid (RNA)**a Structure**

Generally, RNAs are single stranded, however it is possible for some RNAs to adopt secondary and tertiary structures through intramolecular base pairing.

b Function

Cells mainly use RNA as an intermediate support for genes in order to carry out the synthesis of proteins essential to their functions. There are several types of RNA:

- Messenger RNA (mRNA) has the function of carrying genetic information from DNA to ribosomes for protein synthesis.
- Ribosomal RNA (rRNA) is the main constituent of ribosomes and plays an essential role in the process of translation of mRNA into proteins.
- Transfer RNA (tRNA) functions to transport amino acids to ribosomes during the process of protein synthesis.
- Non-coding RNAs (ncRNAs) play a crucial role in the regulation of gene expression, particularly through mechanisms such as microRNAs and RNA interference.

CHAPTER 02 . Insulin

II. Insulin

II.1. Structure

Insulin is a hormone made up of 2 polypeptide chains, an A chain of 21 amino acids, and a B chain of 30 amino acids. These two chains linked together by 2 disulfide bridges located on cysteines in A7-B7 and A20-B19 and an intra-chain disulfide bridge in the A chain in A6-A11 (**Figure 7**). The structure of insulin was determined by Frederick Sanger. This was the subject of the first of his two Nobel Prizes, in 1958.

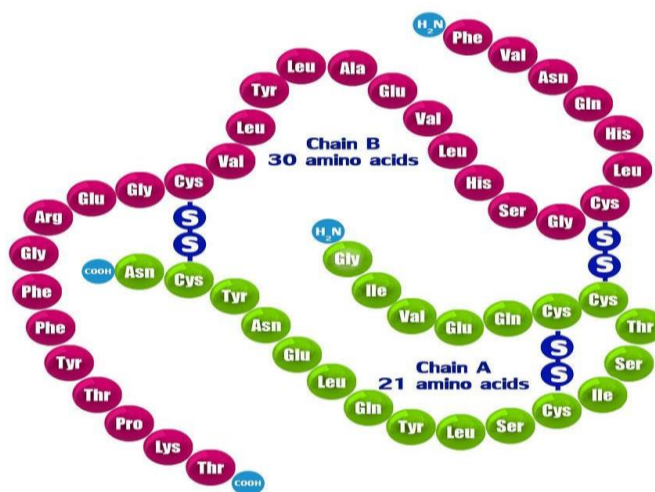


Figure 7. Human insulin structure

II.2. Biosynthesis

Insulin is produced by the β cells of the islets of Langerhans of the pancreas. The biosynthesis of insulin begins in the nucleus of B cells, from the information contained in the genetic code, located on chromosome 11. The synthesis of insulin within beta cells involves the successive cleavage of its two precursors, the pre-pro-insulin and pro-insulin molecules. The gene encoding pre-proinsulin is located at the short arm of chromosome 11. The intracellular pathway continues in the rough endoplasmic reticulum after transcription into RNA of the gene encoding this protein which has a short lifespan. The mRNA translated and exported into the cytoplasm transfers to ribosomes located on the surface of the cisternae forming the complex network of the reticulum. Once synthesized, pre-pro-insulin (98 amino acids) will quickly undergo enzymatic cleavage of 12 amino acids. The signal peptidase which will cleave the signal peptide resulting in the creation of three disulfide bridges. This "pre" segment is cut by enzymes, synthesized by other ribosomes, is responsible for the migration of the protein chain being assembled towards the interior of the cavities of the rough endoplasmic reticulum. The pre-pro-insulin molecule is then transformed into pro-insulin (86 amino acids, molecular weight $\gg$ 9000) containing the chains of amino acids which will give insulin (51 amino acids, molecular weight $\gg$ 6000), plus one segment,

the connecting peptide or c-peptide (31 amino acids, molecular weight $\gg 3000$) connecting the end of chain A to the beginning of chain B. proinsulin, present in the endoplasmic reticulum, will fold back on itself in order to form by alignment the future A and B chains (**Figure 8**). Many proinsulin molecules will then be stored in the form of beta-granules, at the level of the Golgi apparatus. This plays an important role in the exocytosis process. Beta-granules also contain the proteolytic enzymes responsible for the cleavage of C-peptide leading to the formation of mature beta-granules containing an equimolar quantity of insulin and C-peptide. Pro-insulin will undergo the elimination of the C peptide by PC1, which will release a central fragment, while the two newly formed chains will remain associated thanks to the disulfide bridges: finally, the C-terminal end of one of the chains will be cleaved by the action of a carboxypeptidase E (CPE) to become insulin in its mature, and therefore active, form. Proinsulin has a structure very similar to that of the two main growth factors, IGF-1 and IGF-2, and high concentrations of these hormones allow biological effects by signaling after binding to the receptors of the others: hypoglycemia during massive secretion of IGF-1 and IGF-2 by tumors. Insulin circulates at concentrations of the order of nanomoles per liter. Protein C has long been used as a marker of insulin secretion in diabetic patients.

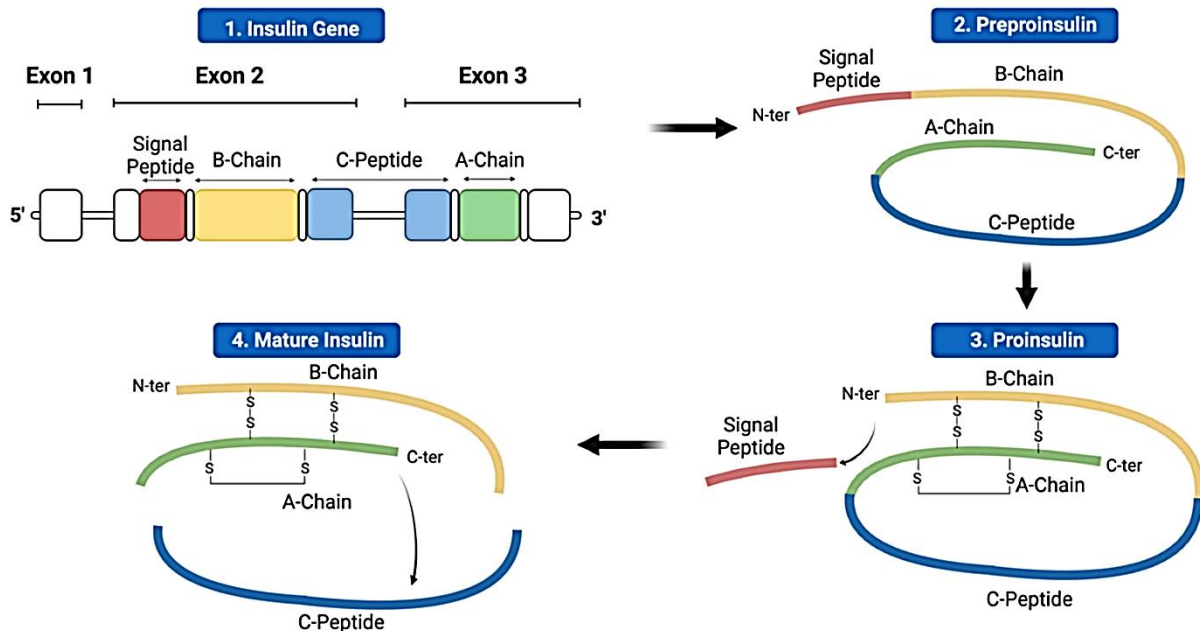


Figure 8. Biosynthesis of Insulin

II.3. Physiology

II.3.1. Secretion

The beta granules mature into a large storage reservoir of insulin, well beyond daily requirements. Insulin is released into the circulation by exocytosis, depending on fluctuations in blood sugar and the plasma concentration of other nutrients (amino acids, fatty acids, ketone bodies). Blood glucose filters through the capillary into the interstitial lymph which bathes the β cells of the islets of Langerhans. The concentration of glucose around the cells is therefore the same as in the blood. The cell imports glucose through a non-saturable transporter GLUT2 (other cells in the body have a receptor that is quickly saturated). The intracellular glucose concentration therefore reflects that of the blood. Glucose is balanced across the plasma membrane thanks to these transporters. The entry of glucose into the beta cell is immediately followed by its phosphorylation by a specific hexokinase; Pyruvate produced by glycolysis preferentially enters mitochondria and is metabolized, containing reducing equivalents in the form of NADH and FADH₂. The transfer of electrons from these equivalent reductants through the mitochondrial electron transport chain is coupled to the pumping of protons from the mitochondrial matrix to the intermembrane space, leading to the generation of ATP. The concentration of the latter is increased in the cytosol, which increases the ATP/ADP ratio. This induces the closure of a potassium channel sensitive to this increase in the quantity of ATP. If the K⁺ (potassium) ions stop coming out, this hyperpolarizes the β cell which is an excitable cell, since it has electrical activity that extracellular glucose concentrations from 5 mmol/l. This depolarization opens voltage-sensitive calcium channels: the opening of the channels favors the entry of calcium and the increase in cytoplasmic Ca²⁺ leading to the exocytosis of insulin granules. Glucose can also receive amplifying signals other than ATP, which plays an important relay in insulin secretion from pancreatic beta cells (Figure 9).

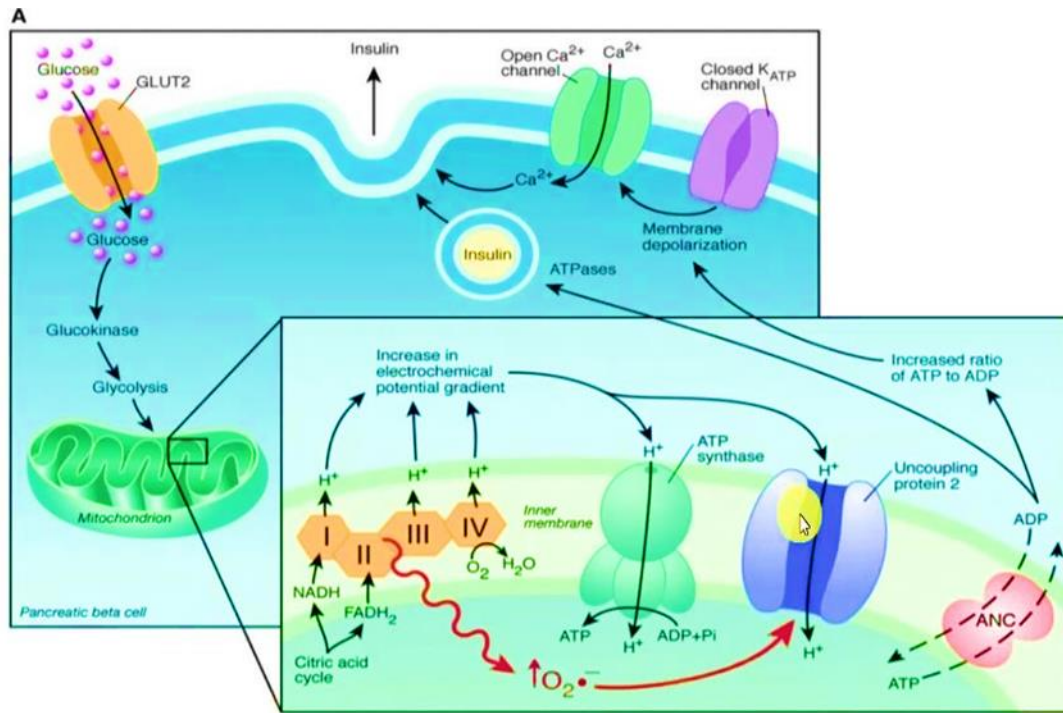


Figure 9. Coupling of glucose with insulin secretion

II.3.2. Distribution

After meals, the objective is to rapidly achieve high insulin levels immediately following food intake. Insulin naturally secretes in the portal vein, or insulin concentrations are significantly higher than those measured during peripheral injection. Therefore, its initial action is directed towards the liver. The concentration of insulin in the portal vein is approximately four times higher than in the peripheral systolic circulation. The only insulin that is released from the liver, approximately 40%, will affect peripheral tissues, including muscle and adipose tissue. This physiological situation cannot be replicated by exogenous insulin, even by an external insulin pump via the subcutaneous route. In fact, insulin diffuses from the site of cutaneous deposit to extrahepatic tissues (peripheral tissues) after injection. Consequently, it is difficult to regulate the initial actions of insulin, particularly the inhibition of hepatic glucose production, and an excess of peripheral effects, particularly on oxidative stress.

Approximately six minutes is the half-life of insulin in the plasma of a normal subject and a diabetic. Its distribution volume is equivalent to the volume of extracellular liquid, which is approximately 20% of the body's weight. This is primarily the monomeric form of insulin that diffuses into the tissues. Insulin can traverse the hemo-endocrine barrier through transporters.

The half-life of proinsulin is 17 minutes and of the C-peptide is 30 minutes.

II.3.3. Catabolism

Insulin has a short half-life, estimated to be between 4 and 6 minutes in the bloodstream after it is released, which enables a precise regulation of metabolism. The decrease in insulin concentration in the blood is the result of two major processes. The first is the degradation of this hormone by enzymes, while the second is a decrease in its secretion by a negative feedback system.

II.3.3.1. Enzymatic degradation

The liver eliminates the circulatory insulin as it passes through the portal circulation, indicating that the portal circulation insulin levels are higher than those in the systemic circulation. The liver is significantly responsible for insulin clearance in the systemic circulation; however, its delayed clearance may result in control issues in individuals with renal disease. Additional degradations may occur within insulin beta granules prior to their release, as well as in other tissues following their binding to the receptor. In this instance, the "insulin-receptor to insulin" complexes are located on the plasmic membrane of the target cell, where they accumulate to the level of invaginations before being internalised by fusion into the endosomes. Subsequently, insulin will be sequestered and enzymatically degraded by one or more proteases that are present in the lumen of endosomes. The enzyme responsible for degradation was first identified in the 1960s. The term "insulinase" is derived from the English term "insulin degrading enzyme." It is a metalloprotease from the zincin family. Its function is to degrade the β -chain. Protein disulfide isomerase (PDI) maintains disulfide bonds. In the end, lysosomes are responsible for the degradation of partially catabolized insulin molecules.

II.3.3.2. Negative feedback control

The process of negative feedback control of the insulin signal may originate from the dephosphorylation of the receptor itself or from the phosphorylation of serine/threonine residues located at the receptor. The negative feedback loop initiated by the binding of insulin molecules to their receptors serves to inform the body that insulin production is no longer required.

II.4. Mechanism of action

Insulin has a crucial role in the regulation of energy metabolism. It is particularly responsible for the utilisation and storage of nutrients in the liver, musculature, and adipose tissue. The beta cells of the Langerhans' islets of Langerhans in the pancreas secrete insulin in response to the physiological or pathological increase in blood glucose concentration. This circulating glucose will induce insulin binding to its receptors, which are primarily located in hepatocytes (liver cells), adipocytes, and muscle cells. Insulin activates the tyrosine kinase domain of its receptor, thereby initiating a series of reactions that will, among other things, result in the translocation of GLUT-4 storage vesicles. In fact, GLUT-4 is a glucose transporter that is stored

in cytoplasmic vesicles in a non-stimulated cell or when insulin concentration is low. The movement of vesicles and their integration at the plasmic membrane level will be initiated by insulin. The concentration of this insulin-dependent transporter in the membrane will subsequently increase (**Figure 10**).

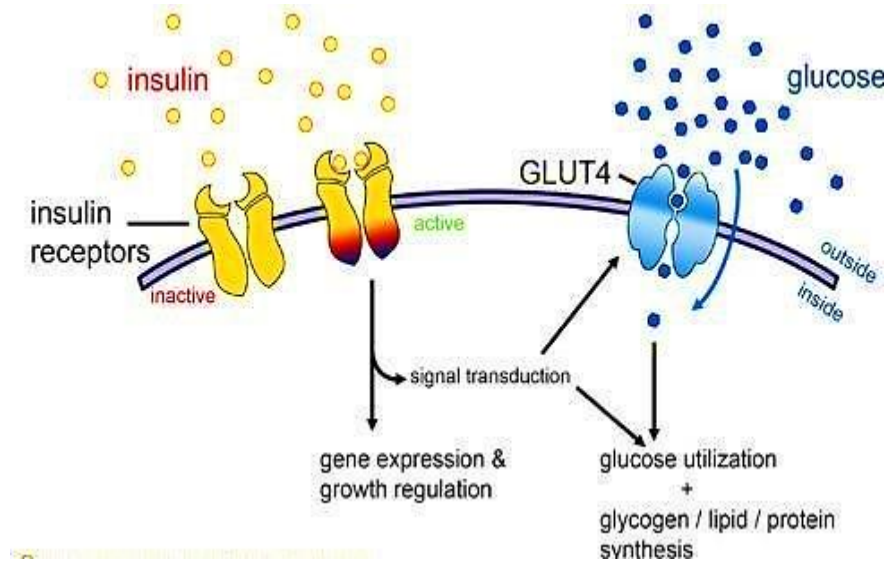


Figure 10. Insulin-induced GLUT-4 translocation and absorption glucose uptake

II.5. Physiological actions

II.5.1. Carbohydrate metabolism

Insulin acts on carbohydrate metabolism by promoting the storage of glucose in the form of glycogen by amplification of glycogenesis in the liver, that is to say the formation of glycogen, by activation of glycogen synthetase allowing its synthesis and by inhibition of glycogen phosphorylase leading to its use. It will also have an inhibitory effect on gluconeogenesis (glucose formation) by stimulating protein synthesis, which will lead to the degradation of amino acids which were until then available for the synthesis of glucose. We therefore see an increase in glycogen stock, particularly in the liver, and a reduction in glucose production in the muscles, adipose tissue and the liver.

II.5.2. Lipid metabolism

Insulin acts on lipid metabolism by reducing their use as an energy substrate and promoting the synthesis of fatty acids from glucose. It stimulates lipogenesis in adipose and hepatic tissues from glucose by stimulating acetyl CoA carboxylase ($\text{acetyl-CoA} \rightarrow \text{Malonyl-CoA}$) and inhibits lipase, responsible for the mobilization of fatty acids.

II.5.3. Protein metabolism

Insulin acts on protein metabolism by promoting the penetration of certain amino acids into the cell, at the same time as the entry of glucose. It stimulates protein synthesis by activating

transcription and translation, and at the same time reduces protein degradation, and in particular their use for gluconeogenesis.

II.6. Production of insulin

II.6.1. History of insulin production

The history of insulin begins with the description of the pancreas by Paul Langerhans in 1869 which highlights the presence of cells grouped into islands. In 1889, Oscar Minkowski and Josef Von Mering discovered the increase in blood sugar levels in dogs after pancreatectomy and demonstrated the link between the pancreas and blood sugar regulation. In 1920 a 29-year-old Canadian surgeon, Frederick Grant Banting (1891-1941), worked on the hypothesis that the pancreas has two distinct functions. The first, already well known, is the exocrine function, that is to say the production of enzymes acting in the digestive tract on digestion. The second is an endocrine function, which means the secretion of a hormone into the blood. This hormone, which would come from the part of the pancreas that was still poorly understood at the time, the islets of Langerhans, would be capable of regulating blood sugar levels. In order to demonstrate his theory, he will attempt to extract and purify the hormone with the aim of using it to treat diabetes. For this he will obtain, with the help of his mentor Dr. John James Rickard Macleod (1876-1935), a renowned professor of physiology at the University of Toronto, a small laboratory as well as 10 dogs for the experiments. He will then be assisted by Charles Best (1899-1978), a young 22-year-old medical student, with a degree in physiology and biochemistry.

In 1921 they tested the first hypoglycemic pancreatic extracts obtained, previously called “soletine”, on dogs rendered diabetic by pancreatectomy. However, the results were not brilliant given the high level of impurities present in this substance whose color was brown. The biochemist James Bertram Collip (1892-1965) then joined the team because it was necessary to isolate a large quantity of insulin, but with fewer impurities. It will thus produce the first refined insulin that can be injected into humans.

Shortly after, still in December 1921, Leonard Thompson, a 14-year-old diabetic boy, was urgently hospitalized at the Toronto General Hospital. His blood glucose was around 5 g/L, he was in ketoacidosis and weighed only 30 kg. Doctors only gave him a few weeks to live since at the time type 1 diabetics ended up falling into a ketoacidotic coma from which they did not recover. In 1922, it was decided to inject humans with a pancreatic extract from the calf. It was Leonard Thompson who was the first to receive several of these insulin injections. His condition will then improve, his weight will increase, his blood glucose will drop to 1.25 g/L and the ketoacidosis will disappear. It was the first time that an injection administered to humans, since 1906 when Dr. Georg Zuelzer had administered extracts of rabbit pancreas to diabetic patients but whose toxicity,

probably due to bacteriological contamination of these extracts, was such that he had to stop the treatment, was working.

The term "insulin", which comes from the Latin "insula" which means islet, was introduced in 1909 by the doctor De Meyer to name this newly discovered substance, produced as was supposed at the time, by the islets of Langerhans. . This name will subsequently be generalized and it is still the one we use today. The Nobel Prize in Medicine was awarded in 1923 to Banting and Macleod for their major advance in the treatment of type 1 diabetes. But Banting judges that Best also played an important role in the work and decides to share the bonus with him, Macleod does the same with Collip

Following the first injections administered to humans, much progress has been made to improve the quality of insulin, which very quickly becomes a marketed drug.

At the beginning, the first insulin marketed, in the form of "ordinary" insulin, was extracted from beef or pork pancreas, imperfectly purified and in the form of an acidic solution at pH 3. Treatment required 3 to 4 injections per day .

The first objective was therefore to purify the product in order to make the injection as safe as possible, then the research, which still continues today, aimed to increase the effectiveness of insulin treatment. In the 1930s, various preparations made it possible to obtain long-acting insulins in order to reduce the number of daily injections, thus significantly improving the living conditions of patients. The term "insulin", which comes from the Latin "insula" which means islet, was introduced in 1909 by the doctor De Meyer to name this newly discovered substance, produced as was supposed at the time, by the islets of Langerhans. . . This name will subsequently be generalized and it is still the one we use today. The Nobel Prize in Medicine was awarded in 1923 to Banting and Macleod for their major advances in the treatment of type 1 diabetes. But Banting judged that Best also played an important role in the work and decided to share the prize with him, Macleod did the same with Collip

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of patients.

Thus in 1935, Hans Christian Hagedorn (1888-1971) combined insulin with protamine, which made it possible to extend the resorption time under the skin. Scott and Fisher discovered the delaying effect of zinc and in 1936 they developed a slow-acting insulin, by adding zinc to protamine insulin. This process, which induces crystallization of insulin, further extends its action time. This insulin is called IPZ for “Insulin Protamine Zinc”. Insulin containing only zinc will be developed a few years later by the Dane Hallas-Moller

In 1946, when marketed insulin was in the form of an acidic product, researchers at the Hagedorn Institute (Denmark) developed the first insulin with a neutral pH. This is NPH for “Neutral Protamine Hagedorn”, an intermediate-acting insulin, which is still widely used today. Frederick Sanger (1918-2013), an English biochemist, described for the first time the biochemical structure of the insulin molecule in 1955. Insulin was then the first protein whose chemical structure could be fully determined. He received a Nobel Prize for this work in 1958.

II.6.2. Production of insulin from bovine and porcine pancreases

The initial method of producing pancreatic extract is to apply the purification technique. From freshly minced beef or pig pancreas, the extract is produced following a series of steps. The organs were dissected free of adherent fat and connective tissues, cut into small pieces, and homogenized in an acidified alcohol solution. The use of acidified alcohol for insulin extraction protects against enzymatic degradation since under such condition's insulin is known to be highly soluble whereas proteolytic enzymes and other contaminating proteins exhibit very low solubilization. The homogenate was stirred for 3 hours slowly, centrifuged to separate the alcoholic extract from most of the insoluble materials. The pellet was re-extracted for approximately 2 hours with alcohol. The mixture was centrifuged and the first and second extracts were pooled (added together) and made basic ($\text{pH} = 8$) with ammonium hydroxide. Collip had discovered that the active ingredient was found entirely in the alcohol, which formed the upper layer observed in the centrifuge test tube.

After separation of the protein-containing supernatant by centrifugation, soluble proteins can be neutralized using either ammonium hydroxide (NH_4OH), calcium carbonate (CaCO_3) or calcium sulfate (CaSO_4). The next extraction steps can be carried out by evaporation of ethanol or precipitation of the extract with ammonium acetate ($\text{CH}_3\text{COONH}_4$) allowing the solubilization of fatty substances and the precipitation of proteins. The heavy inactive precipitate which has formed is eliminated by filtration on a wooden filter press. The filtrate was acidified with sulfuric acid. The filtrate was concentrated in vacuo, then the concentrate was filtered through paper to remove fats and other insoluble materials. After defatting the extract, crude insulin can be obtained

by adding high concentrations of salts (NaCl) which make it possible to separate the insulin from non-protein compounds as well as certain contaminating proteins and polypeptides. The precipitate then obtained by filtration, then dissolved in distilled water and concentrated in order to remove all traces of alcohol and obtain the desired concentration of the active ingredient, and the mixture was acidified to pH = 1, 8 – 2.2 with hydrochloric acid. By stirring for approximately 2 hours, the active ingredient is dissolved.

Crude insulin can be further purified by repeated crystallization and filtration. Additionally, purification steps may also consist of various chromatographic techniques, including gel filtration, ion exchange system, and high-performance liquid chromatography (HPLC).

II.6.3. Production of insulin by biotechnology

Rosalyn Yalow (1921-2011) was also awarded a Nobel Prize, awarded once again to researchers working on insulin, in 1977 for her work with notably Solomon Berson (1918-1972) which led to the development a radio-immunological assay method in the 1950s. This technique allows the precise dosage of substances present in tiny quantities in a liquid medium. Initially developed for the measurement of insulin in the plasma of diabetics, the technique was quickly extended to the measurement of multiple substances, peptide and non-peptide. In particular, it made it possible to demonstrate that insulin extracted from the pancreas of animals contained impurities and was responsible for the formation of antibodies directed against insulin, causing more or less local allergic reactions or even lipodystrophy.

Following this, it was realized that the insulin molecule was species specific. There were therefore differences between human insulin and injected animal insulins from pork and beef. Pharmaceutical laboratories will thus embark on the chemical synthesis of insulin in order to overcome the compatibility problems encountered with animal insulin. In 1965 chemical synthesis became possible simultaneously in the USA, Germany and China. In 1978, Eli Lilly laboratories successfully cloned the human insulin gene for the first time. This is a fundamental step in the production of insulin by genetic engineering. Industrial production of human insulin then becomes possible. Two methods were used in the 1980s:

- Semi-synthesis (Novo Nordisk): pig insulin only differs from human insulin by one amino acid. By modifying it using chemical processes, we can then give it the structure of human insulin.
- Biosynthesis (Lilly): in this case, we insert the human gene coding for insulin into a bacteria or a yeast and, by multiplying, these microorganisms will produce insulin which will then only have to be purified. The first insulin produced by biosynthesis was marketed in 1982. This manufacturing process, which no longer depends on an animal source, provides unlimited quantities of insulin and is still used today.

II.6.3.1. Semi-synthetic production from pig insulin

These insulins constituted an advance compared to animal insulins, but they are no longer marketed today.

a Structure

Compared to the structure of human insulin, animal insulins have a similar structure but have certain differences in the amino acid sequence. Indeed, pork insulin differs from human insulin by one amino acid on the B chain at position 30 and beef insulin has three different amino acids compared to human insulin located on the A chain at position 30. 8 for the first, on the same chain in position 10 for the second and on chain B in position 30 for the third (**Table 1**).

Table 1. Differences between human and animal insulin sequences

Species	A8	A10	B30
Human	Threonine	Isoleucine	Threonine
Beef	Alanine	Valine	Alanine
Porc	Threonine	Isoleucine	Alanine

b Principles

The process consists of making a semi-synthesis from pig insulin which only differs from human insulin by the alanine in B30 in place of threonine (human insulin) to obtain an insulin identical to that of the human; this protocol was developed in 1978 and used by Novo in 1982.

c Preparation

The extracted porcine insulin will undergo hydrolysis by 2 possible means:

Transpeptidation or coupling by carboxypeptidase A or by achromobacter protease to obtain desalanine (B30)-porcine insulin or DAI. Then, a coupling of the DAI with the compound containing the threonine to be grafted (Thr – Obut) is carried out via trypsin (concentration 1-5 μ molar), at pH7 and at a temperature below 12°C for 10-20 hours, which allows a yield greater than 90%.

Finally, the insulin ester obtained is isolated on a chromatographic column of Sephadex G50 and DEAE-Sephadex A25. Human sequence insulin is released by hydrolysis by trichloroacetic acid in the presence of anisole with a yield greater than 50%.

In order to improve yields, trypsin is fixed on the chromatographic columns in which the solution is passed 10 times to the DAI stage, which makes it possible to obtain insulin yields greater than 80%. The synthesis can also be carried out in a single transpeptidation step with the threonine-containing compound (Thr-OBu) after hydrolysis of porcine insulin by trypsin, resulting in a human insulin ester. Finally, the chemical structure of semi-synthetic insulin is identical to that of human insulin described by Sanger (**Figure 11**).

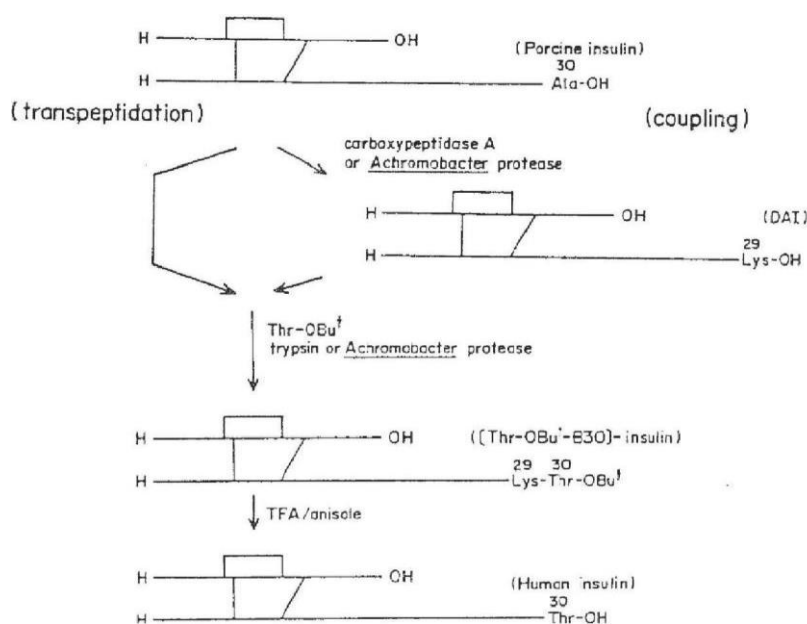


Figure 11. Steps of semi-synthetic production from pig insulin

In general, the use of semi-synthetic insulin obtained from the pancreas of animals has been beneficial for diabetics because the effectiveness has been improved due to the structural homology with endogenous insulin and the reduction of immunological reactions.

Chromatography combined with enzymes such as trypsin has made it possible to obtain high yields of human insulin.

This process did not, however, eliminate the animal origin and it was necessary to continue to obtain supplies of animal pancreases. This led to continued research to find another mode of production with the aim of:

Have human insulin directly

Overcome animal sources and specific problems linked to organ harvesting in slaughterhouses.

The development of the production of human insulin by genetic engineering has solved both problems.

II.6.3.2. Production of fully synthetic recombinant insulin

The production method is that of genetic engineering. The Lilly laboratories associated with Genentech have been using it since 1978, but it was not until 1982 that human biogenetic insulin was marketed for the first time under the name Humulin®.

This type of insulin production does not depend on animal raw materials, which considerably reduces costs. Biosynthesis is carried out using bacteria (*Escherichia coli*) or yeast (*Saccharomyces cerevisiae*) and there are 2 production methods (**Figure 12**)

a Production from peptides A and B

One of the methods of manufacturing insulin consists of synthesizing and isolating the gene (DNA) consisting of nucleotide sequences coding for the A protein chain and the gene coding for the B protein chain of insulin; then these 2 genes are inserted separately into a bacterial strain of *Escherichia coli* via a plasmid associated with the β -galactosidase gene, which leads to the formation of chain A and chain B, these 2 chains are then purified and combined in the laboratory to produce recombinant human insulin.

b Production from proinsulin

This method consists of isolating the mRNA of the insulin precursor proinsulin then transforming it into DNA via reverse transcriptase and inserting this genetic material coding for proinsulin into the *Escherichia coli* plasmid which will be cloned and purified. Proinsulin is converted to insulin by proteolytic enzymes. The insulin obtained by these two processes then undergoes numerous purification steps.

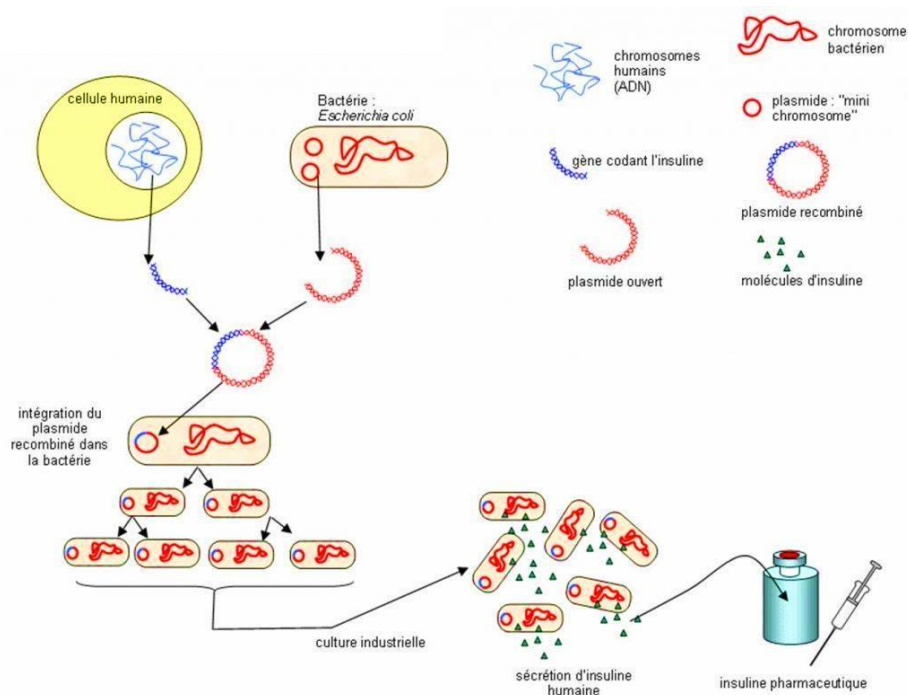


Figure 12. Insulin production using genetic engineering

c Production of insulin analogues

In the mid-1990s, researchers began improving the way human insulin works in the body by changing its amino acid sequence and creating analogues, chemicals that mimic another substance well enough to trick the cell. There are several insulin analogues. Instead of synthesizing the exact DNA sequence for insulin, manufacturers synthesize an insulin gene where the sequence is slightly modified which will directly produce the desired analog. At the end of the manufacturing process, additives are added to the insulin to produce the desired type of insulin. Additives vary between brands of the same type of insulin. The following table summarizes the different modifications to obtain the desired analogues (**Table 2**).

Table 2. Insulin's analogues

Analogues	DCI	Modifications
Fast action	Lispro Insulin	Inversion of position of lysine B29 and proline B28
	Aspart Insulin	Proline B28 has been replaced by aspartic acid
	Glulisine Insulin	Aspartic acid in the B3 position of human insulin is replaced by lysine, and lysine at position B29 is replaced by glutamic acid
Prolonged action	Glargine Insulin	The addition of 2 charges + (2 arg) on the C -terminal of chain B. This modification increases the pHi to 6.7 which makes the molecule more soluble in a slightly acidic medium and less soluble at physiological pH. The replacement of asparagine A21 with glycine to avoid deamidification and dimerization of insulin because its injectable solution is acidic (pH4).
	Detemir Insulin	Rapidly absorbed into the blood, binds to albumin from where it is then only slowly released. Removal of threonine at position 30 of the chain B, acetylation of lysine at position 29. The added fatty acid allows insulin to bind to albumin
Intermediate action	protamine isophane Insulin	a suspension obtained by mixing insulin human and protamine in the presence of small amounts of zinc and phenol (or meta cresol)
Action lente et ultra-lente	Zinc depot insulins	It is a mixed zinc insulin suspension: 70% in crystallized form and 30% in amorphous form.

CHAPTER 03. Interferons

III. Interferons

III.1. Historical

Interferons (abbreviated IFN) are glycoproteins of the cytokine family (signaling molecules controlling the immune system). They were discovered in 1957 by Isaacs and Lindenmann, who noticed that chicken cells infected with the influenza virus produced a factor that allowed other cells to become resistant to the virus. This factor was named interferon, because it allows viral interference, that is to say the acquisition of resistance to a virus by a cell. It was subsequently revealed that this factor was in fact composed of different proteins from the same family, which were discovered to play varied roles in vertebrates.

III.2. Classification

According to their antigenic and physicochemical properties. IFNs are classified into 3 types: types I and III are involved in innate antiviral immunity in most cells of the body, while type II plays a preponderant role as a communication molecule between specialized cells of the system. immune. These proteins are heterogeneous and vary depending on the inducing agent and the producing cell. Within the same species, there are a variety of IFNs. This diversity has led to the establishment of a classification according to the producing cell, physicochemical and antigenic properties.

III.3. Diversity

Type I interferons are themselves divided into several subtypes: IFN- α and β , the most common and most studied, but also IFN- ω , ϵ and κ , which are less studied and whose action is limited to certain organs, unlike IFN- α and β which are almost ubiquitous in the body.

Type II includes only IFN- γ , which plays a role very similar to other cytokines, that is to say mainly a communication function between cells of the immune system.

Type III includes IFN- λ , recently discovered and whose biological functions are still debated. However, it seems that their functions are very close to those of IFNs. Type I: IFN- λ induces a non-specialized state of antiviral resistance and uses the same transduction pathway as type I IFNs, with the exception of their receptor.

Humans have 21 genes encoding interferons: 13 for IFN- α , 3 for IFN- λ , and only one for each of the β , ω , ϵ , κ and γ classes. The grouping of several genes in the same subtype is due to the very high homology of their sequences: we say that these genes constitute a multigene family.

III.4. Structure

Cloning and sequence analysis of the IFN γ gene detailed sequence. The IFN γ cDNA encodes a polypeptide of 166 amino acids, 20 of which represent the signal peptide cleaved during the process of secretion of mature IFN γ by T lymphocytes. The sequence thus obtained is different from that of IFN α and β unlike

the IFN genes α and β which are located on chromosome 9 and which do not have introns. However, the IFN γ gene is located on chromosome 12 with 3 introns. Interferons α and β , type I interferons, have a common structure composed mainly of five alpha helices. Although the monomers of each are very similar in structure, the functional form of both is a dimer, and the two dimerize differently, IFN α 2a along homologous surfaces and IFN β 1 on opposite sides of the protein. IFN γ is represented in its dimerized form (**Figure 13**). This representation of the three-dimensional structure highlights the numerous alpha helices that compose them.

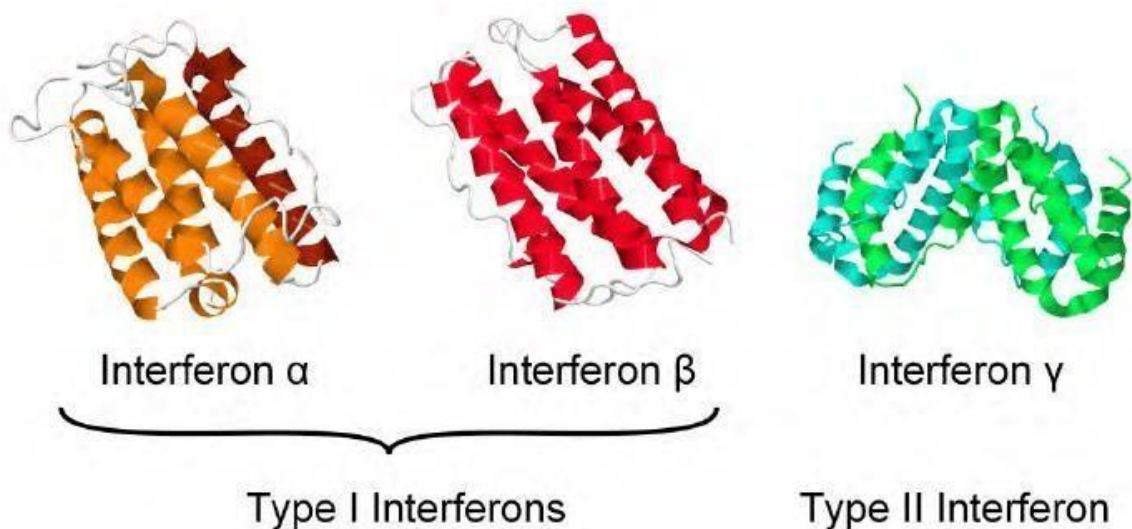


Figure 13. Structure and type of interferons

III.5. Biosynthesis

In vertebrates, almost all cells are capable of producing type interferons. Those of type III are produced and induce a state of antiviral resistance in many cell types, but seem rather specific to tissues highly exposed to viral infections, such as mucous membranes. Finally, it should be noted that specialized immune cells capable of producing phenomenal quantities of interferons in response to a viral infection have been identified: plasmacytoid dendritic cells or pDCs. These blood cells produce up to 10,000 times more IFN- α than other cells and, as such, are called IPCs (interferon producing cells).

The production of interferons is activated by the perception of signals indicating a viral infection. This perception is ensured by PRRs (pattern recognition receptors), a large class of host proteins that recognize PAMPs (pathogen-associated molecular patterns) presented by pathogens. This immune response is innate, that is to say, immediately mobilized upon the first encounter with a given pathogen. It does not require a long period of “education” of the immune system as is the case for the so-called adaptive immune response which is characterized by the production of

antibodies and lymphocytes highly specific to the pathogen.

PAMPs can be nucleic acids present in a particular cellular compartment: for example, the presence of double-stranded RNA or DNA in the cytosol indicates to the cell that it is infected, because, physiologically, the cells of Vertebrates do not produce free double-stranded RNA, and confine their DNA to their nucleus or mitochondria. However, many viruses either have a double-stranded RNA or DNA genome which is released into the cytosol during an infection, or produce such a molecule in the cytosol during their replicative cycle. Their genome is then replicated and expressed directly in the cytosol. These molecules stimulate the innate immune response and in particular the production of interferons. PAMPs include other molecules.

The PRRs involved in the interferon response belong, for the most part, to two protein families: TLRs (Toll-like receptors) and RLRs (RIG-I-like receptors). TLRs 3, 7, 8 and 9 are involved in the recognition of viral nucleic acids present in the extracellular environment or in endosomal pathways. TLR3 is a receptor located at the plasma membrane or at the membrane of endosomes (organelles responsible for recycling or degradation of endocytosis products; they constitute the entry zone into the cell for many viruses), which recognizes double-stranded RNA located in endosomes or the extracellular environment. When it binds its ligand, it activates TRIF, an adapter protein, which in turn activates IRF3. This protein then migrates into the nucleus and induces the expression of genes encoding interferons. Similarly, TLR 7 and 8 recognize single-stranded RNA in endosomes and the extracellular environment, and TLR 9 is activated by DNA present in endosomes.

Likewise, the signaling pathways activated by many PRRs converge on IRF3 or IRF7, which induce the synthesis of interferons. This synthesis can also be induced through members of the RLR family, notably RIG-I and MDA5, in response to the presence of viral RNA in the cytosol. Finally, a heterogeneous family of cytosolic DNA receptors, including in particular the cGAS/STING couple, has been characterized in recent years, and allows the induction of an interferon response in cells infected by a DNA virus (**Figure 14**).

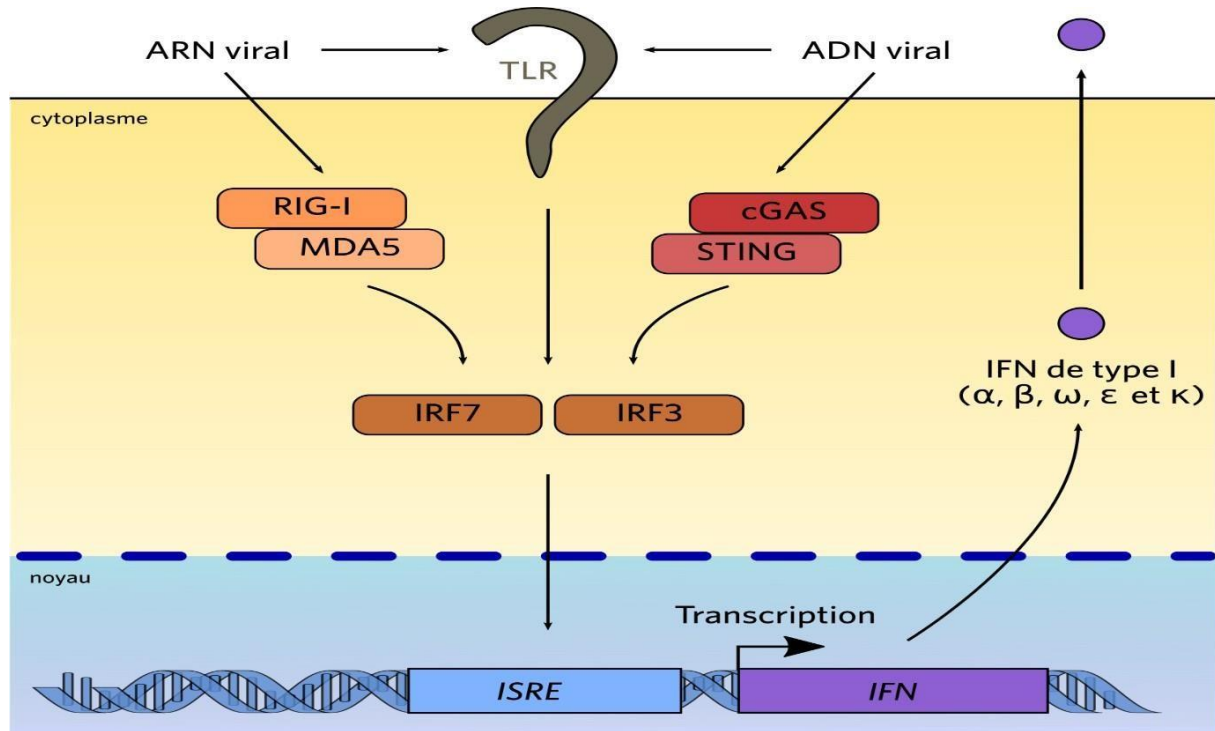


Figure 14. Mechanisms of production of type I interferons

Viral DNA and RNA are PAMPs that can activate several types of PRR: here represented TLR, RIG-1 (an RLR) and cGAS. Via adapter proteins, these PRRs activate IRF3 or IRF7, which induce the production of interferons, which are secreted into the extracellular environment.

III.6. Mechanism of action

As intercellular communication molecules, IFNs are small proteins that are secreted by producing cells and diffuse into the extracellular environment. They attach to their receptors when they encounter them, which induces an intracellular signaling cascade. Interferons α and β have a common receptor, which in the activated state is a heterodimer of the IFNAR1 and IFNAR2 subunits. λ interferons bind to a receptor specific to them.

The mechanism of their action is complex and not yet completely understood. After binding to specific receptors on the cell surface, interferon activates the Jaks/Stat pathway and leads to the expression of numerous genes.

The binding of an IFN by a membrane receptor, then in the monomeric state, causes its dimerization with another monomer, which causes a change in their respective conformations, and consequently the activation of a protein which is linked to the receptor, a JAK family kinase. One of the monomers is JAK1, the other is TYK2. These two proteins activate each other when the two receptor subunits dimerize. and then phosphorylate STAT proteins, which are free cytosolic proteins. Phosphorylation of STAT proteins promotes their dimerization, which causes a conformational change revealing nuclear localization sequences. The STAT1/STAT2 dimer can

then bind to the consensus sequence that it recognizes, called ISRE (interferon-stimulated response element). This sequence is found in the promoters of several genes, which are activated by binding of the STAT dimer. This transduction pathway is called the JAK/STAT pathway. Type I and III IFNs activate approximately 300 to 400 genes, called ISGs (interferon-stimulated genes), which ensure a complete antiviral response targeting all stages of the replicative cycles of different viruses (**Figure 15**).

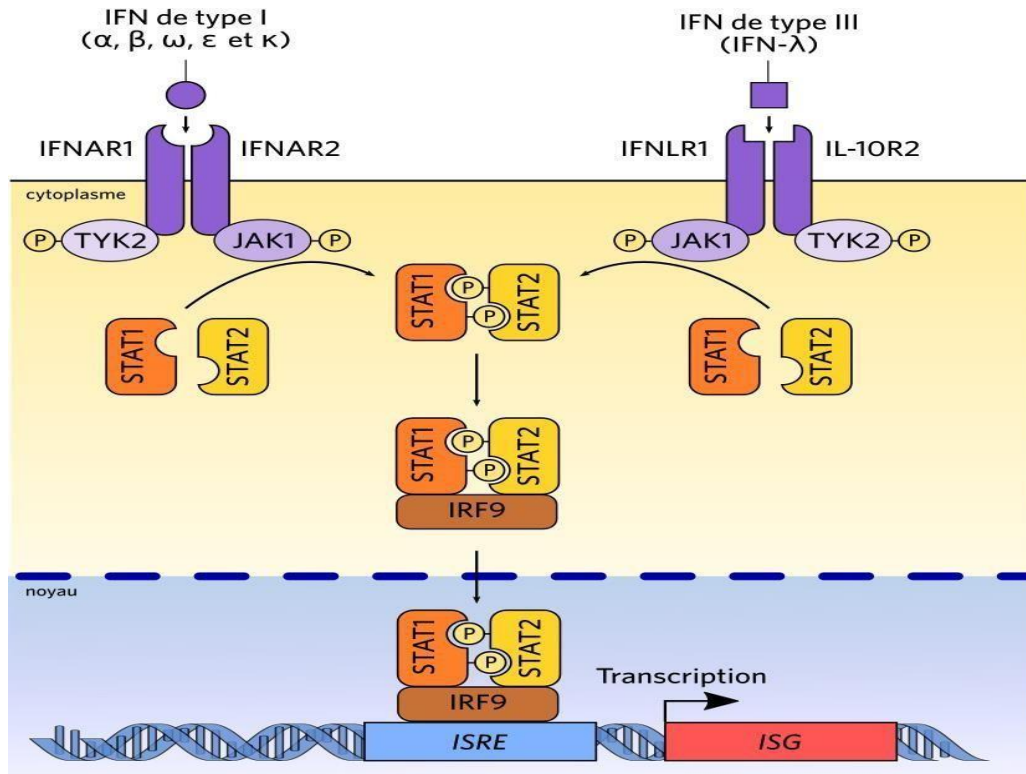


Figure 15. Type I and III interferons and JAK/STAT signaling pathway

Binding of an IFN results in activation of the receptor's JAKs, which phosphorylate the receptor and become self-phosphorylated. Thus activated, they phosphorylate STAT proteins, which dimerize, associate with the accessory protein IRF9 (thus forming the ISGF3 complex) and relocate in the nucleus, where they induce the expression of ISGs (interferon-stimulated genes). The activation cascade is normally "turned off" once an infection is cleared to avoid damage to uninfected cells. However, this activation state is not reduced to normal levels in people with SLE, where a higher level of interferon is present. This higher amount of interferon is also measurable by an increase in interferon-stimulated gene expression seen in lupus patients, called the interferon response signature.

III.7. Physiological roles

After release, interferon causes an increase in the expression of the two major histocompatibility complexes (CMHI and CMHII) for the presentation of viral peptides to T cells, which can then lead to the activation of other cells to kill infected cells and eliminate them. It also increases intracellular levels of double-stranded RNA-dependent protein kinase or PKR which recognizes nucleic viruses and activates RNase to degrade viral RNAs. PKR also slows protein synthesis by inactivating translation initiation factors, so that viral protein synthesis is slowed. PKR also plays a role in important mechanisms such as apoptosis and the induction of genes (including that of interferon itself). P53 is also activated, which is pro-apoptotic. Interferons activate immune cells, particularly natural killer cells and macrophages. They activate the Mx proteins, which associate with the HIV capsid, and, through a still unknown mechanism, would thus prevent its replication; inhibiting the fusion of viral membranes with cellular membranes. In addition, other genes activated by IFNs lead to overexpression of PRRs, reinforcing the vigilance of cells to danger signals, or repress the translation of new proteins, which prevents the virus from producing its proteins and allocate the majority of cellular resources to antiviral defense (the mRNAs coded by the ISGs escape translational repression). In addition, some of the ISGs are themselves transcription factors that activate or inhibit other genes, ensuring amplification of the interferon signal. The interferon response is thus diversified in its targets and has a considerable amplitude, since almost all the activity of the cell is modified, and now largely devoted to its defense. 2'-5' oligo-adenylate (2-5A) synthetases as well as the associated 2-5A system, double-stranded RNA-dependent protein kinase or PKR, nitric oxide synthase, major histocompatibility complex proteins and ubiquitin-related protein. Other proteins with antiviral potential, such as PML proteins, will also be mentioned. Understanding the mechanism of action of these different proteins allows the development of new antiviral strategies. These are discussed, along with the current clinical effectiveness of interferon in viral conditions pathogenic to humans, such as human immunodeficiency virus infections, hepatitis B and C viruses, and Epstein-Barr and human herpes virus type 8.

Finally, let us note two other functions of type I and III interferons: on the one hand, the interferon response can induce apoptosis (programmed cell death) of the target cell; on the other hand, these interferons have a role as modulators of the adaptive immune response. Thus, IFN- α and β participate in the differentiation of certain T lymphocytes and IFN- λ is involved in the maturation of T and B lymphocytes.

III.8. Production of interferon

Interferons are extracellular proteins involved in signaling which, like interleukins, belong to the cytokine family. They can be produced following viral infections and have the property of inducing a state of resistance in cells to many of them. They also have antiproliferative, antitumor and regulatory properties of immune functions and differentiation processes. For these reasons, interferons are used clinically in the treatment of various viral infections and neoplasias.

III.8.1. Production from human cells

III.8.1.1. Preparations containing HuIFN- α

a IFN from Leukocyte

Until recently, human leukocytes (white clots) obtained from donor blood were the source of most of the interferon used. Briefly, blood is collected on anticoagulant and centrifuged. The “white clot” cells, which collect at the interface between plasma and erythrocytes, are separated and resuspended in fresh isotonic saline containing human serum. They are stimulated to form interferon by adding an inducing virus, generally the Sendai virus, cultivated in chicken eggs. Interferon production is completed within 18 hours, and crude interferon is separated from leukocytes by centrifugation. This crude interferon was generally purified by precipitation using potassium thiocyanate acid and extraction of the precipitate by alcohol with fractional reprecipitation at different pH values. The purified interferon (P-IF) preparations thus obtained have a specific activity of approximately 3×10^6 international units (IU) per mg of protein (they represent approximately 1% of pure interferon protein) and the yields are approximately 5 million IU for each white clot from a single 0.45 l blood donation. This technique for preparing leukocyte interferon is simple in principle and does not require complicated equipment. However, experience has shown that it requires considerable attention to detail. Leukocytes collected for therapeutic purposes from leukemia patients have also been used as a source of interferon.

b IFN from lymphoblastoid cells

Many transformed human cells can produce interferons when properly stimulated. Lymphoblastoid B cells of a particular line, Namalwa, were used as a source of large amounts of HuIFN- α . These cells are stimulated by the Sendai virus in the same way as the leukocytes of the white clot, but they offer the advantage of being able to multiply and produce interferon in suspension culture. Containers with a capacity of up to 4000 L have been used on an industrial scale, allowing for more economical production. In one system, Namalwa cell interferons are routinely processed to 80-95% purity. This product contains at least 8 subtypes of HuIFN- α , which differ in their chemical, physical, antigenic and biological properties. Large clinical trials using this lymphoblastoid cell interferon formulation are underway.

III.8.1.2. Preparations containing HuIFN- β

Fibroblast interferon, HuIFN- β , is obtained from cultured fibroblasts; these are diploid cell strains from newborn foreskins or embryonic tissues and certain continuous cell lines (3, 4, 5,). To date, the best results have been obtained by treating these cells with synthetic ribonucleic acid, polyribonucleosinic-polyribocytidylic acid, in the presence of metabolism inhibitors, actinomycin and cycloheximide, added in a precise order over time. . The IFN- β thus prepared can be purified in various ways and the preparation that was used in clinical trials contained 106 IU or more per mg of protein. Since fibroblasts only multiply when they adhere to a surface, special technical processes have been developed to provide the large surface area needed for mass culture. Even under these conditions, production on an industrial scale continues to be relatively difficult.

III.8.1.3. Preparations containing HuIFN- γ

Human white clot cells provide HuIFN- γ when stimulated by lectins, staphylococcal enterotoxins, or other mitogens. T cells are thought to be the source of interferon. Crude preparations often contain various lymphokines as well as HuIFN- α and - β , but purification is relatively difficult because the molecule is unstable. Until now, there has been no communication on the use of this type of interferon in patients.

III.8.2. Production by recombinant DNA techniques

Production in bacteria and yeast is a way to prepare a single IFN protein at what could be a relatively low cost.

III.8.2.1. Preparation of HuIFN- α

The genes corresponding to HuIFN- α were cloned with *Escherichia coli* (prokaryotes) and yeast (eukaryotes) bacterial cells. A group of researchers managed to obtain the expression of 6 different genes corresponding to interferons, and very high yields of interferon protein were reported. Preparations of particular subtypes, mainly HuIFN- α 2 (recombinant A) and HuIFN- α 1 (recombinant D), have been purified to 80—95% and used in clinical trials. The different cloned IFN- α subtypes are likely to have different biological activities.

III.8.2.2. Preparation of HuIFN- β and HuIFN- γ

Human β and γ interferons can also be obtained from bacterial, yeast and animal cells by recombinant DNA techniques. While native IFN- β and IFN- γ are glycoproteins, those produced by bacterial cells do not contain a carbohydrate. At present, there is little published information about the biological characteristics of these interferons and nothing is known about their clinical behavior.

CHAPTER 4. Erythropoietin

IV. Erythropoietin

Erythropoietin (EPO) is a hormone naturally produced in our body, it is key to the regulation of erythropoiesis. This powerful anti-anemic agent could only be used in human therapy from 1989 thanks to industrial progress and the development of biotechnologies. Initially used in the treatment of anemia of chronic renal failure, EPO has seen the number of its indications increase over these two decades: anemia of cancers, solid tumors but also autologous transfusion programs and orthopedic surgery scheduled major

IV.1. Historical

Forty years after the discovery of America by Christopher Columbus, Pizzaro noticed, during the battles against the Incas, greater valor among the soldiers who had previously stayed on the high plateaus. It was then necessary to wait until 1863 for, following the work of Antoine Lavoisier on oxygen and the work of German physiologists including that of Hope-Seiler on the role of red blood cells in the transport of oxygen, to be noticed by a French doctor Denis Jourdanet an identity of symptoms (asthenia, pallor, dyspnea, headaches) between anemic patients and polyglobulinic inhabitants of the Mexican highlands. He attributes the symptoms observed to the decrease in the oxygen content of red blood cells and therefore of the blood, which he calls anoxemia.

This observation was taken up a few years later, in 1882, by a student of Claude Bernard, Paul Bert, in his work on "Barometric Pressure", which remains one of the foundations of aeronautical medicine.

In 1890, E. Viault noted, during an expedition to Peru, "the considerable level of red blood cells in the blood of the inhabitants of the highlands of South America" and the polycythemia occurring in his traveling companions, more or less important depending on the altitude and the length of stay.

In 1906, Paul Carnot and his assistant C. Deflandre demonstrated a clear increase in the number of red blood cells in healthy rabbits to which they had previously injected serum from an anemic rabbit; They deduce that this increase is mediated by a circulating substance that they call hemopoietin, present in the blood of anemic rabbits in response to hypoxia, but this work will not be continued. The existence of hormonal regulation of erythropoiesis will be confirmed in 1943 by Krumdieck repeating the Carnot experiment, then by Kurt Reissmann in 1950 using a cross circulation experiment between the rat and finally by Erslev in 1953. The latter highlights evidence the occurrence of frank reticulocytosis in healthy rabbits perfused with large quantities of serum from a rabbit made anemic by bleeding, thus confirming the action of this hormone on erythropoiesis. In 1948, E. Bonsdorff named this hormone erythropoietin.

IV.2. Structure

EPO is a highly glycosylated 30.4 kD glycoprotein, encoded by the long arm of chromosome 7. The mRNA derived from the EPO gene must undergo some modifications before being transported into the cytoplasm and translated into protein. Translation takes place at the level of ribosomes, forming a protein of 193 amino acids. As the molecule found in the bloodstream contains only 165 amino acids, certain post-translational modifications take place. EPO is a secreted hormone, so there is a leader peptide that is deleted, leading to a deletion of the first 27 amino acids. In addition, for an as yet unknown reason, there is loss of a carboxyterminal arginine carried out by intracellular enzymes. This therefore restricts the size of the protein to 165 amino acids, for a molecular weight of 18 kDa. **Figure 16** represents the primary structure of human EPO. We can notice the presence of four cysteines linked in pairs (Cys 7-161 and Cys 29-33) by disulfide bridges. These are necessary for the normal biological activity of the molecule.

The mature EPO molecule is heavily glycosylated: three N-glycosylation sites (on the asparagines at positions 24, 38 and 83) and one O-glycosylation site (on the serine at position 126). The structure of the glycosylated chains is not identical in all natural erythropoietin molecules.

These four carbohydrate chains each contain between two and four sialic acid molecules. There are thus several isoforms of EPO, defined by the number of sialic acid molecules (between 4 and 14), their biological behavior, the affinity for their receptor and their half-life.

The diversity of the motifs of the 4 carbohydrate chains fixed on the protein skeleton of erythropoietin causes the molecular mass of human EPO to vary from 26 kDa to 33 kDa, the mass of 30 kDa corresponding to the most frequent form.

EPO is a cytokine which has the particularity of not being produced by cells of the immune system. That said, it shares a common secondary structure with many cytokines, within the IL-2 (interleukin type 2) subfamily.

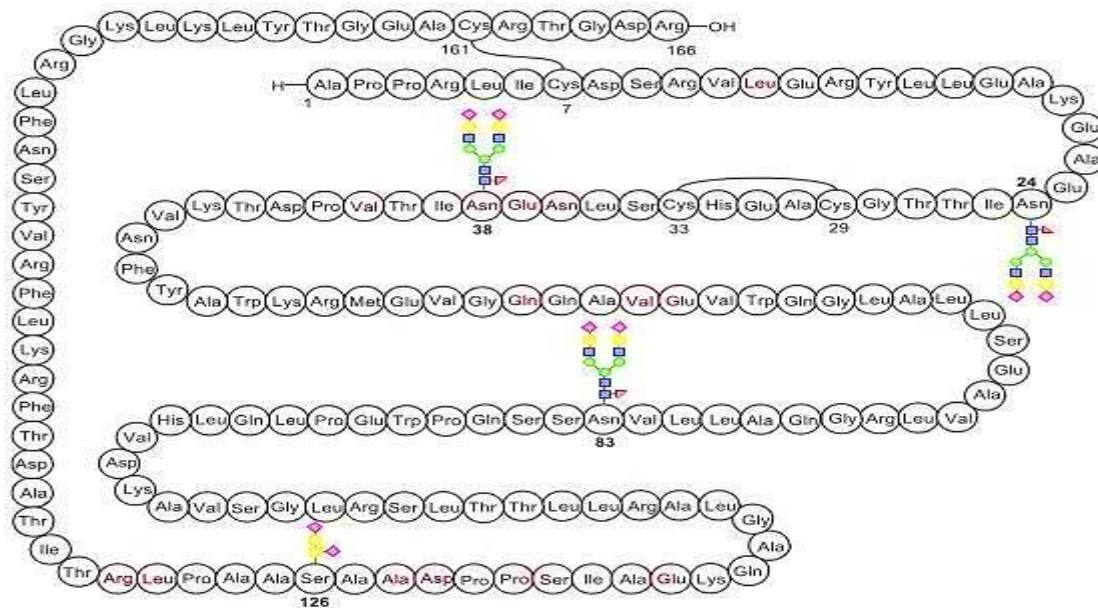


Figure 16. Primary structure of Erythropoietin

IV.3. Biosynthesis sites

Not having the property of being stored at the cellular level, EPO must be produced on demand in a more or less acute manner depending on the prevailing situation. Under these conditions, the production of EPO is strongly induced during massive blood loss, essentially leading to the main stimulus, tissue hypoxia. To counter this oxygen deficit, increased production of EPO is essential because it will lead to proliferation. erythrocyte, allowing the situation to be restored. Whether during these latter circumstances or for the replacement of senescent erythrocytes. Several organs have demonstrated the ability to produce EPO.

Before birth, EPO production occurs only in the liver. It is the non-parenchymal cells and the hepatocytes which adjust their level of EPO synthesis according to the needs felt. For this purpose, the tissue hypoxia detection system essentially consists of an oxygen gradient at blood level. As it moves towards the central hepatic vein, the oxygen-rich blood becomes increasingly defective, and these are the cells (non-parenchymal and hepatocytes) surrounding this central vein which sense this lack

IV.3.1. Kidney

Hypoxia is the stimulus that triggers the production of EPO in the producing organs: essentially the kidney and liver. The cells producing EPO in the kidney were identified in 1988 as a subpopulation of peritubular fibroblastic interstitial cells located in the renal cortex and the outer medulla. However, some authors have described EPO production by proximal tubular cells. However, the production of EPO by these cells requires efficient renal function. Renal production of EPO is constitutive and according to an “all or nothing” law. The increase in EPO production is achieved by increasing the number of producing cells. The tubular cells play a role as an O₂

sensor and transmit O₂-dependent signals to neighboring interstitial cells, inducing EPO production.

IV.3.2. Liver

A small production (10%) is provided by liver cells in adults (while in the fetus, this liver production ensures, at the beginning, all of the EPO production).

The precise identification of the liver cells involved in this production was more difficult due to the lower quantity of EPO produced by this organ. Two distinct cell populations seem to secrete the hormone: a majority contingent of hepatocytes distributed around the centro-Iobular veins of the liver and a smaller contingent of interstitial cells located in a perisinusoidal position in the spaces of Disse. There are numerous analogies between these cells and the fibroblast interstitial cells of the kidney. These cells are less sensitive than kidney cells to hypoxia but they can individually vary their rate of EPO production.

IV.3.3. Other EPO production sites

EPO messenger RNA was detected by RNase protection test in very hypoxic rats in the testes, spleen and finally the brain. The physiological significance of these results is unknown, the quantities of EPO produced are negligible compared to the renal and hepatic contributions.

IV.4. Receptors and mechanisms of action

Red blood cells emanate from a pluripotent hematopoietic stem cell (HSC) which will engage in a myeloid differentiation pathway from a mixed progenitor called CFU-GEMM (for Colony Forming Unit Granulocyte/Erythrocyte/Megakaryocyte/Macrophage). The CFU-GEMM will then differentiate towards a restricted progenitor in the erythrocyte pathway, the BFU-E (for Burst Forming Unit Erythropoies; CFU-E derive from BFU-E. They have a capacity for self-renewal (60 to 80% phase Go) and reduced proliferative capacity.

Their survival and proliferation depend on several cytokines, in particular EPO. EPO-Rs are present in greater numbers on the surface of CFU-E.

The CFU-E will proliferate and differentiate in successive stages to lead to the formation of precursors of the erythroblastic lineage.

The EPO receptor (EPOR) belongs to the receptor tyrosine kinase superfamily. It is an asymmetric transmembrane complex of 508 amino acids (55 to 78 kDa) which belongs to the cytokine superfamily.

The extracellular domain carries the catalytic domain. It includes 2 immunoglobulin-like domains (D1 and D2) composed of a β -sandwich structure containing 7 β strands. The distal part of the D1 domain and the proximal part of the D2 domain are connected by a short hinge. The D1 domain is stabilized by 2 disulfide bridges via the 4 cysteine residues. D2 contains a conserved

WSXWS motif.

To stimulate erythropoiesis, EPO produces a proliferative signal on red blood cell precursor cells: BFU-E and CFU-E (**Figure 17**). The regulation of erythropoiesis by EPO is enabled by EPOR receptors, located on the membrane surface of the progenitors of the erythrocyte lineage. It is the CFU-E progenitor which has the most receptors on its surface, hence its high sensitivity to EPO. The number of receptors decreases as cell differentiation progresses. Thus, the reticulocyte and the mature erythrocyte no longer carry EPOR. By binding to its receptor, EPO changes the conformation of the receptor which dimerizes leading to the activation and phosphorylation of the JAK-2 protein which binds to the intracellular domain of the receptor. Then, JAK-2 phosphorylates the EPO receptor on the tyrosine residues of the intracellular domain which allows different signaling molecules to bind to these sites such as: STAT-5 (signal transducer and activator of transcription 5) and PI3 - K (phosphatidyl-inositol 3-kinase). STAT-5 activates transcription of target genes while PI3-K inhibits apoptosis (a process of cell death). It seems well established that the pathways activated by the EPO receptor allow cell proliferation and survival through the activation of PI3-kinase.

A major action of EPO-EPOR (EPO receptor) binding is the increase in intracellular calcium levels via IP3 (inositol 3-phosphate). Once the EPO is linked to its receptor, 60% of the internalized EPO is released while 40% is degraded intracellular level. On the other hand, it remains to be discovered whether in vivo the resecreted EPO is biologically active (**Figure 18**).

BFU-E results in the terminal formation of red blood cells in 10 to 20 days, CFU-E in 5 to 8 days. A Proerythroblast, following 4 mitoses, produces on average 16 red blood cells. The acidophilic erythroblast which expels its nucleus becomes a reticulocyte. The newly formed reticulocyte remains in the bone marrow for 48 hours, then passes through the spinal sinusoids and ends up in the peripheral blood where it loses its ribosomes in less than 48 hours to become a mature red blood cell. The synthesis of red blood cells is continuous and harmonious, it is estimated at 200 billion per day, it allows the maintenance of physiological standards (4 to 5×10^{12} /l in women, 5 to 6×10^{12} /l in men). Red blood cells have an average lifespan of 120 days.

To stimulate erythropoiesis, EPO produces a proliferative signal on red blood cell precursor cells: CFU-E. It notably promotes the transformation of CFU-E into proerythroblasts and the transformation of the latter into erythrocytes. Many other factors are involved in erythropoiesis. Among them: androgens, iron and vitamin B6 (hemoglobin formation) and vitamins B9 and B12 (DNA formation).

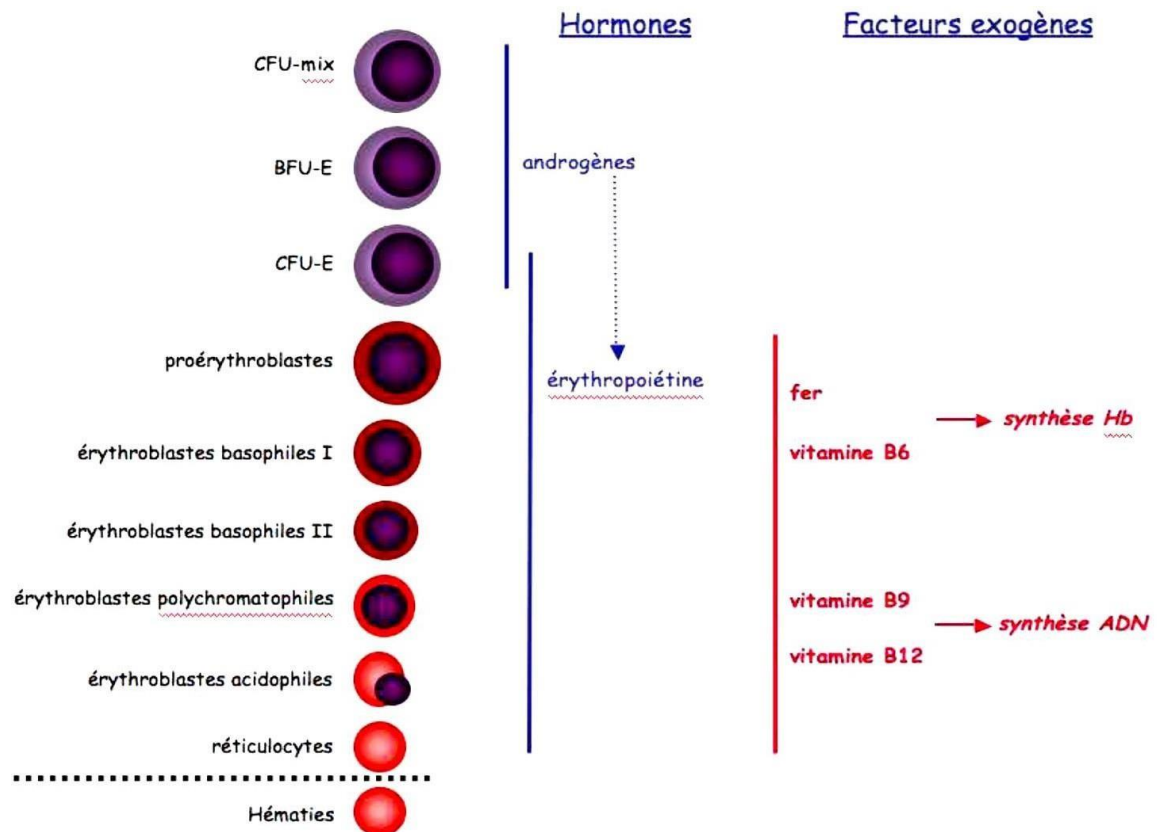


Figure 17. Regulation of erythropoiesis by erythropoietin.

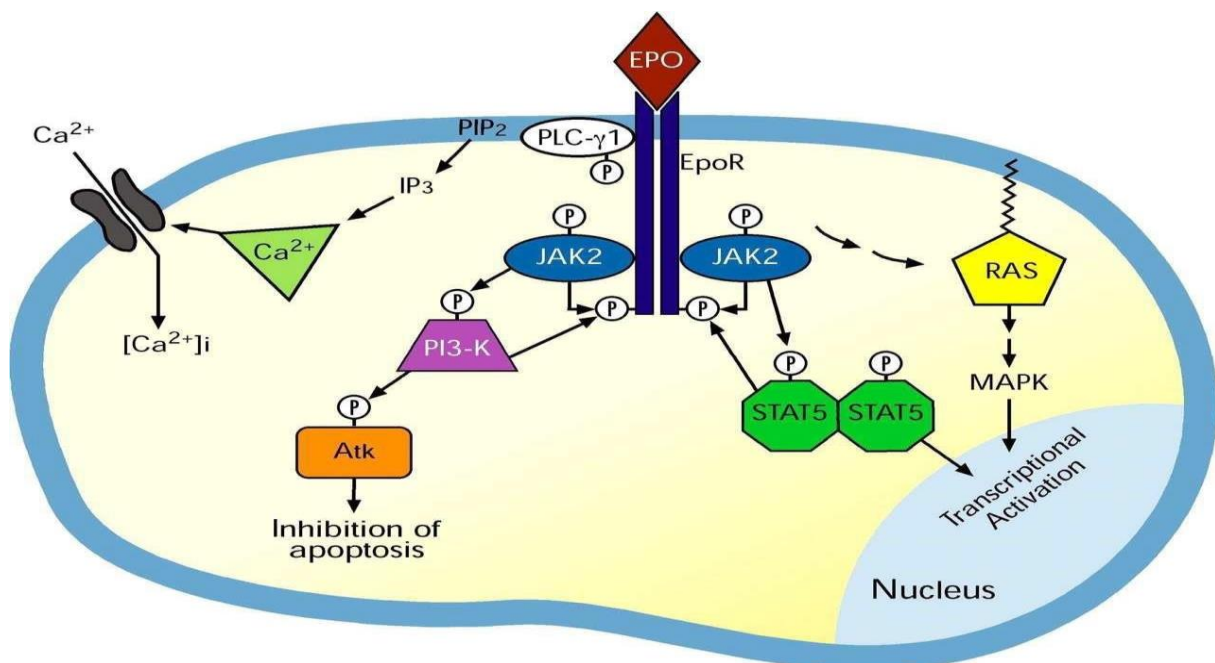


Figure 18. Simplified diagram of signal transduction induced by EPO by binding to its receptor EPOR.

IV.5. Structure/function relationship in the EPO molecule

The sugar motifs that this glycoprotein carries play an important role in the function of the molecule. The ablation of sialic acids located at the ends of the glycosylated EPO chains (whether urinary or synthetic EPO) increases the activity of this molecule *in vitro* but abolishes its activity *in vivo*. The digestion of human urinary EPO by an endoglycosidase causing the elimination of oligosaccharides fixed by N-glycosylation at Asn 24, 38 and 83 leads to a total disappearance of EPO activity *in vivo* while it persists and even increases *in vitro*.

In the absence of sialic residues, the speed of elimination of the molecule by the body increases. EPO devoid of its sialic acids would bind to liver cells, at the level of sugar receptors, through its galactoses, N-acetylglucosamines and mannoses thus exposed, leading to its rapid metabolism by these liver cells. Furthermore, the presence of oligosaccharide chains could prevent EPO molecules from forming aggregates between them, aggregates which reduce the biological activity of the molecule. Concerning the affinity with the receptor, it has been shown that the EPO molecule lacking the oligosaccharides fixed at N on the asparagines but still possessing the sialic acids of the chain fixed at O on the serine, presents an increase ($\times 2$) in its affinity with the EPO receptor and that this increase is even stronger ($\times 4$) if all the sialic acids have been removed. Sialic acids would therefore hinder binding to the EPO receptor. We can remember that the protein part of the EPO molecule is sufficient for its binding to its receptor and that the sugars protect the molecule from its inactivation in the body before it reaches its target cells, but hinder its attachment to the receiver.

IV.6. Physiology

The secretion of this hormone is influenced by oxygenation levels. Hypoxia causes a significant increase in EPO production following the activation of a transcription factor, the hypoxia-inducible factor HIF-1 (Hypoxia Inducible Factor 1 α) (**Figure 19**). Erythropoietin production is greatly increased in hypoxia. This phenomenon is made possible thanks to the presence, on the EPO gene, of a 3' activation site to which a transcription activator complex will attach. Oxygen-dependent regulation of gene expression is mediated by a hypoxia-induced transcription factor, HIF-1 α . When HIF-1 α is activated, it associates with HIF-1 β , also called ARNT (aryl hydrocarbon receptor nuclear translocator) to form the HIF-1 heterodimer. The amount of cytoplasmic ARNT is independent of the oxygen content of the cell. On the other hand, the HIF-1 α subunit is only detectable in the hypoxic cell. The HIF-1 heterodimer then forms a complex with the transcriptional cofactors P300, CBP and HNF-4 which binds to the 3' activation site of the EPO gene and activates its transcription.

In the oxygenated cell, heme-functional proteins such as NADPH oxidase generate

superoxide ions by electron transfer which are converted to reactive oxygen species (ROS). The HIF-1 α factor is in turn oxidized by these species then recognized by the proteasome which degrades it. In the hypoxic cell, HIF-1 α is not oxidized and can associate with the ARNT factor to form the HIF-1 dimer which is then translocated to the nucleus to activate the expression of hypoxia-inducible genes, such as that of EPO. The hypoxic stimulus results in the appearance of EPO-specific mRNA within 1 hour.

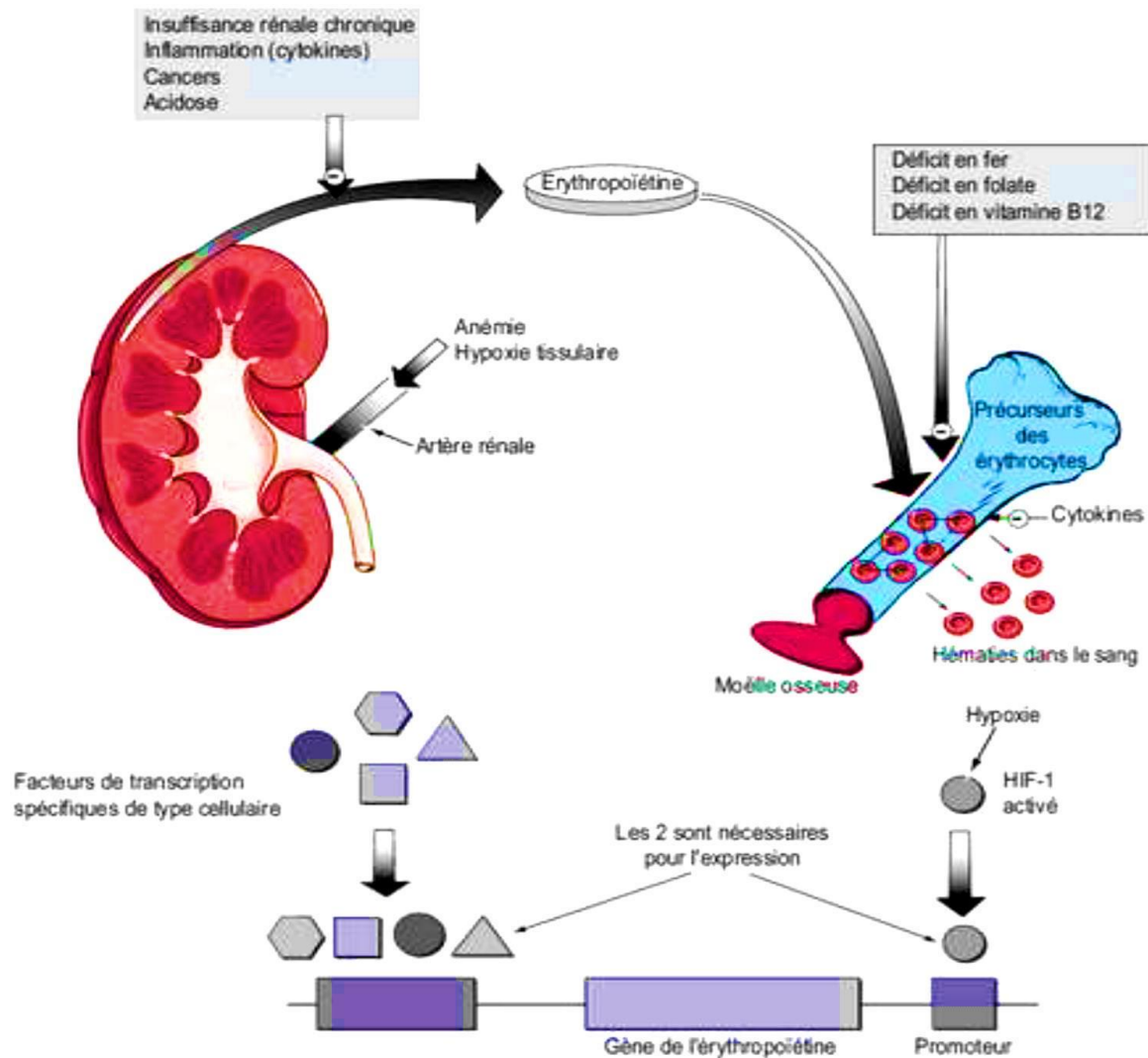


Figure 19. Diagram of the erythropoietin creation cycle.

Hypoxia causes a significant increase in EPO production following activation of the transcription factor HIF-1. In the kidney, CKD, inflammation, cancer or acidosis can inhibit the production of erythropoietin. In the bone marrow, deficiencies in iron and vitamins B9 or B12 can inhibit the formation of erythrocytes. Conversely, to avoid excessive production of red blood cells, erythropoiesis must be negatively regulated. This regulation is essentially carried out by the level of circulating EPO as we have just explained. More recently, it has just been demonstrated that the

negative regulation of erythropoiesis is carried out by a paracrine mechanism involving death receptors such as FAS. In this model, it was proposed that erythroblasts at the end of differentiation (polychromatophils and acidophils) in the bone marrow, within the erythroblastic islets, express FAS ligand and thus interact directly with the progenitors and earlier erythroblastic precursors expressing the FAS receptor in order to induce maturation arrest and apoptosis of these progenitors and precursors. Thus, the level of “mature” erythroblasts in the marrow could retroactively control erythropoiesis. The half-life of EPO is quite short (4 to 7 hours). Few data on EPO metabolism are available. It is likely that EPO degradation occurs after enzymatic cleavage in the liver of terminal sialic acids from glycans. In healthy subjects, the elimination of EPO takes place mainly in the liver via the Gal receptor. Renal elimination of EPO is less significant.

IV.7. Production

In 1957, L. Jacobson highlighted the role played by the kidney in the synthesis of erythropoietin. In 1977, human erythropoietin was purified from the urine of anemic patients by Miyake and Goldwasser and the first radioimmunological assays were possible from 1979.

In December 1983, Amgen's Californian genetic engineering team managed to clone the human erythropoietin gene from the hormone purified by Goldwasser and whose amino acid sequence he knew. Unfortunately, the urinary concentration of the hormone is extremely low, making any industrial production unfeasible.

From 1985, the team of Kenneth Jacobs and his collaborators, simultaneously that of Fu-Kuen Lin, succeeded in expressing the gene by mammalian cells transfected with the human EPO gene. This genetic recombination process makes it possible to obtain in unlimited quantities a pure glycoprotein analog of endogenous EPO: recombinant human erythropoietin or r-HuEPO. Industrial production of erythropoietin can then begin with the first therapeutic indication being the treatment of anemia in patients suffering from chronic renal failure. The first tests are reported in the *Lancet* of November 22, 1986 by Christopher Winearls et al. and in the *New England Journal of Medicine* of January 8, 1987 by Joseph Eschbach et al.

The extraction of erythropoietin for therapeutic or research purposes can be done by different methods, whether from natural animal sources or by biotechnology techniques.

IV.7.1. Production from animal sources

IV.7.1.1. Blood plasma

The production of EPO from blood plasma is a complex and demanding process. Despite its current lesser use due to advances in biotechnology allowing the production of recombinant EPO, this method remains a significant and historical example of protein purification. The usual steps in this process are:

a Collection of blood plasma

Plasma is collected from whole blood, which is obtained from human or animal donors. Then the plasma is separated by centrifugation to remove blood cells.

b Preparation of blood plasma

Plasma frequently undergoes processing to remove unwanted proteins and concentrate erythropoietin. The techniques used include protein precipitation by adding salts such as ammonium sulfate or organic agents to cause protein precipitation. The plasma is cooled, then the precipitating agents are added gradually to separate the proteins according to their solubility.

c Centrifugation and filtration

Centrifugation and filtration are used to remove precipitates and cellular residue. Dialysis removes small molecules and salts from plasma, while leaving larger proteins, such as EPO, in the plasma. the solution.

d Chromatography

Chromatography is an analysis method used in chemistry. It represents the essential process for separating erythropoietin (EPO) from other proteins present in plasma. Various forms of chromatography are implemented successively in order to achieve a high degree of purity

- Affinity chromatography

This technique involves using columns equipped with specific ligands that bind to erythropoietin (EPO). These ligands may consist of antibodies specifically targeting EPO or EPO receptors attached to a matrix. The plasma passed through the column then, EPO binds to ligands, while other proteins pass through

- Ion exchange chromatography

This technique involves using columns to fractionate proteins based on their charge. Then, EPO is purified from the column by adjusting the salt concentration or pH of the elution buffer.

- Gel filtration chromatography

This technique involves sorting proteins based on their size. EPO, being of a specific size, is isolated from other proteins of larger or smaller size.

e Concentration and final purification

Ultrafiltration consists of using membranes to concentrate EPO while eliminating small impurities and salts. While diafiltration consists of replacing the buffer in the solution in order to stabilize erythropoietin and eliminate potentially denaturing agents.

f Validation and verification of data

Biochemical methods such as SDS-PAGE gel electrophoresis and mass spectrometry are used to assess the integrity and authenticity of EPO.

Biological activity tests are designed to ensure the biological activity of EPO. These tests may include in vitro experiments conducted on bone marrow cell cultures to assess stimulation of red blood cell production.

g Formulation and storage

Erythropoietin is formulated in an appropriate excipient to ensure its long-term stability. Conservation is generally carried out in freeze-dried form or in solution, at appropriate temperatures to avoid spoilage.

IV.7.1.2. Urine

This method is historically used, notably for the first isolations of EPO, but is less common today due to the low concentrations of EPO in urine and the difficulty of purification.

Production by recombinant DNA techniques

Purification of erythropoietin from blood plasma is a laborious and expensive process, involving several purification steps to achieve the required purity and potency. Modern biotechnology techniques, such as the production of recombinant EPO in mammalian cells, have largely replaced this method due to their efficiency and ability to produce large quantities of high purity EPO.

IV.7.2. Production of recombinant human erythropoietin

IV.7.2.1. Mammalian cells

Since the molecular cloning of the EPO gene, it is possible to use it to produce synthetic EPO by genetic recombination. The molecule produced is called rHuEPO (recombinant human EPO). Mammalian cells are essential for producing EPO, because this molecule contains a large carbohydrate portion (40% of its mass) which cannot be constructed by the cellular machinery of a bacteria.

The production of rHuEPO by genetic engineering is commonly done by transfer of the human EPO gene into Chinese hamster ovary cells (CHO, for Chinese ovary cells), or human kidney cells, which will produce, in culture, protein. The genetic code being universal, the nucleotide sequence of the gene encoding the amino acid sequence of the protein will allow the production of an identical chain of amino acids, regardless of the cell that produces it. On the other hand, glycosylation is not encoded by the gene itself, and these post-translational modifications may depend on the cell type in which biosynthesis takes place.

Erythropoietin (EPO) is produced in the culture medium and then harvested. The purification of EPO is carried out using chromatographic techniques similar to those used for its extraction from plasma.

IV.7.2.2. Insect cells

The baculovirus-insect system involves the insertion of erythropoietin (EPO) genes into a baculovirus, which is subsequently used to infect insect cells such as Sf9 cells. Infected cells synthesize EPO, which is then purified from the culture medium using chromatographic techniques similar to those used for its extraction from plasma.

IV.7.3. Different human recombinant EPOs

Recombinant human EPO (rHuEPO) derived from eukaryotic cells has a peptide sequence identical to that of EPO of urinary origin. The molecular mass of the protein part is 18,400 Da, with 4 glycosylation chains: 3 N-glycosylation at Asparagine 24, 38 and 83, and one O-glycosylation at serine 126. It can present up to 14 sialic acids. . The molecular mass of the glycosylated protein is 30.4 kDa. The glycosylated units, representing 40% of the total mass of the glycoprotein of the molecule, are necessary for the in vivo biological activity of EPO.

IV.7.3.1. Epoetins

Epoetins are recombinant human erythropoietins composed of the same amino acid sequence as endogenous EPO. However, their glycosylation, essential for biological activity in vivo, varies depending on the transfected cell strain in which EPO is synthesized as well as physiological factors including the culture conditions to which these cells are subjected [174]. Thus the different types of epoetin currently available commercially contain a greater proportion of sialic acid residues than endogenous EPO and differ slightly from each other.

a Epoetin- α

EPO α has a structural topology similar to other members of the cytokine family. It is composed of 4 α helices: A α (residues 8 to 26) located in front of D α (residues 137 to 161), and B α of smaller size (residues 56 to 83) located in front of C α (residues 90 to 112).

The method of administration is by subcutaneous or intravenous injection. This requires frequent injections. The intravenous route is preferably used in hemodialysis patients (Elliott S, et al, 2008).

After subcutaneous injection, the half-life of EPO α at the plasma level is approximately 20 hours. In the context of anemia linked to CKD, the dosages of EPO α are 50-100 Units/kg three times a week. EPOGEN® will be marketed in 1989 by the Amgen laboratory and EPREX® in 1990 by the Janssen-Cilag laboratory.

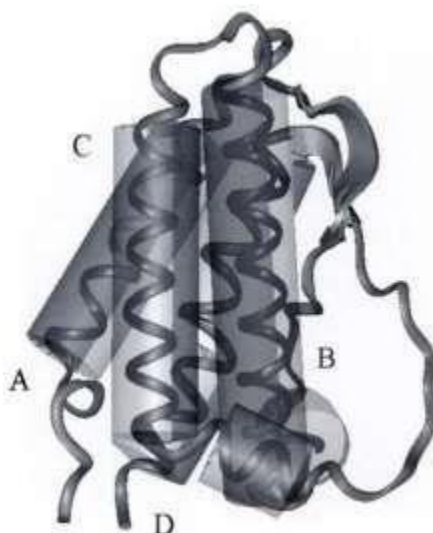


Figure 13. Quaternary representation of EPO α

b Epoetin- β

In the same way as EPO α , it is synthesized from the ovaries of Chinese hamsters. Compared to EPO α , EPO β contains a higher quantity of basic isoforms (Storring et al 1998) and a greater number of tetra-sialylated oligosaccharides (around 46%) (Deicher and Hörl, 2004).

EPO β has a longer half-life than EPO α (24 hours) after subcutaneous injection with a frequency similar to that of EPO α (3 times per week) (Agarwal et al 2008). EPO β was first developed and produced in 1990 by Boehringer Ingelheim under the name Recormon. In 1998, Roche acquired Boehringer Mannheim, making Roche the world number 1 in the diagnostics industry. Following its acquisition, RECORMON® was replaced by NEORECORMON®.

c Epoetin- ω and epoetin- δ

Epoetin- ω is produced by a different cell strain than the other two epoetins, so the glycosylation and pharmacokinetic properties of this molecule are different. Epoetin- ω is produced in BHK (Baby Hamster Kidney) cells.

Unlike epoetins derived from CHO cells, only 60% of the molecule is O-glycosylated on the serine at position 126. The majority of the oligosaccharide chains are 4-branched and a greater proportion of the carbohydrates are tetrasialylated compared to epoetins. Endogenous EPO. In addition, one of the N-glycosylated sites contains a phosphorylated residue on part of the chain. Small clinical studies have found that epoetin- ω may be slightly more potent than epoetin- α . But these clinical data remain to be explored further.

In March 2002, the European authorities approved the marketing by the Shire laboratory of a new erythropoietin in the treatment of anemia of chronic kidney failure. Epoetin- δ also called EPO gene activator (DYNEPO®) is produced by human cells, genetically modified to transcribe and translate the EPO gene under the control of a new regulatory DNA sequence. The introduction

of this new sequence was carried out by homologous recombination of non-coding areas of the DNA. The term “gene-activation” actually means the control of gene expression by the introduction of new promoter sequences making it possible to encode native DNA sequences.

IV.7.3.2. Darbepoetin-a (NESP)

Compared to EPO, human or recombinant, the new erythropoiesis stimulating protein (NESP) is made up of a fixed sequence of 165 amino acids, close to that of EPO, on which are grafted in addition the 3 N- chains glycosydic chains in position 24, 38 and 83 and the O-glycosydic chain in position 126 of EPO, 2 N-glycosydic chains in position 30 and 88. The positions of these 2 additional glycosylation chains were chosen and defined so as not to not modify the ternary structure, and therefore the binding capacities to the EPO receptor.

This required a change in the amino acid sequence, since N-glycosylation can only occur on an asparagine-amino acid x-serine/threonine sequence. Alanine at 30 and tryptophan at 88 of EPO were therefore replaced by asparagine; histidine in 32 and proline in 90 were replaced by threonine and proline in 87 by valine.

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