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Abdelhafid Boussouf
University - Mila

جامعة المجاهد عبد الحفيظ
بوالصوف- ميلة

Faculty of Natural and Life Sciences

كلية علوم الطبيعة والحياة

www.univ-mila.dz

Handout of Biochemistry and Physiology for Second-Year Master's Students in Applied Biochemistry

Prepared by :

Dr. AMIRA Aicha

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Preface

Since the mid-twentieth century, the study of metabolism has evolved from the characterization of major pathways and enzymatic assays to the application of molecular, immunological, and integrative approaches. These advancements have enabled the analysis of metabolic regulation at cellular, organ, and organismal levels, linking metabolic changes to gene regulation and cellular processes, and highlighting their relevance in health and disease, including conditions such as diabetes, cancer, genetic disorders, immune dysfunctions, and microbiota interactions.

This document provides course support for biochemistry and physiology, intended for second-year Master's students specializing in Applied Biochemistry. The handout follows the teaching program approved by the Ministry of Higher Education and Scientific Research.

The course is structured into four main chapters. The first chapter focuses on the study of various metabolic pathways, while the second chapter examines the regulation of these pathways. The third chapter introduces essential biochemical techniques used in the analysis of biomolecules. Finally, the fourth chapter emphasizes the application of enzymatic and immunological tools, along with the investigation of pathophysiological variations observed in diverse biological and clinical contexts.

Fiche matière

Semestre : S3

Intitulé du master : Biochimie appliquée

UEF1 : Biologie, physiologie et Régulations métaboliques

Intitulé de la matière : Biochimie et physiologie

Crédits : 6

Coefficients : 3

Objectifs de l'enseignement

Posséder les connaissances fondamentales de biochimie structurale et métabolique et de physiologie animale.

Connaissances préalables recommandées

Etre capable d'intégrer l'ensemble des métabolismes et leur régulation dans une dynamique des systèmes physiologiques. Maîtriser les techniques biochimiques liées au diagnostic clinique.

Contenu de la matière : Biochimie et physiologie

- Etude des voies métaboliques
- La régulation des voies métaboliques et leurs déviations - Exploration fonctionnelle
- Etude des techniques biochimiques
- Utilisation des outils enzymatiques et immunologiques - Etude des variations physiopathologiques

Mode d'évaluation :

Evaluation continue + examen final

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CHAPTER 1 : STUDY OF METABOLIC PATHWAYS

1. Introduction to metabolism

Metabolism consists of a series of reactions that occur within cells of living organisms to sustain life. The metabolic process involves many interconnected cellular pathways to ultimately provide cells with the energy required to carry out their function (Judge and Dodd; 2020).

A metabolic pathway consists of a series of enzymatic reactions that produce specific products. The term metabolite refers to a substrate, intermediate or product in metabolic reactions (Satyanarayana and Chakrapani; 2013).

Metabolism is divided into two main pathways:

- **Catabolism :** Catabolism releases energy because it is an oxidative process. The overall catabolic pathway is cellular respiration, where glucose and other nutrients are broken down under aerobic conditions. Ingesting food provides us with fuel for respiration, which is converted into CO2 and H2O, giving us energy , which is summarised here (Hanselius and Eldemark; 2010):

Organic compounds + oxygen → carbon dioxide + water + energy (ATP + heat)

Catabolism occurs in three steps (figure 1) (Satyanarayana and Chakrapani; 2013) :

Step 1 : Conversion of complex molecules into their constituent elements ; Polysaccharides are broken down into monosaccharides, lipids into free fatty acids and glycerol and proteins into amino acids.

Step 2 : Formation of simple intermediates; the constituent elements produced in step (1) are degraded into simple intermediates such as pyruvate and acetyl CoA.

Step 3 : Final oxidation of acetyl CoA; Acetyl CoA is completely oxidized to CO2, releasing NADH and FADH2 which are finally oxidized to release a large amount of energy (in the form of ATP). Krebs cycle (or Citric Acid Cycle) is the common metabolic pathway involved in the final oxidation of all energy-rich molecules. This pathway receives carbon compounds (pyruvate, succinate, etc.) derived from carbohydrates, lipids, or proteins.

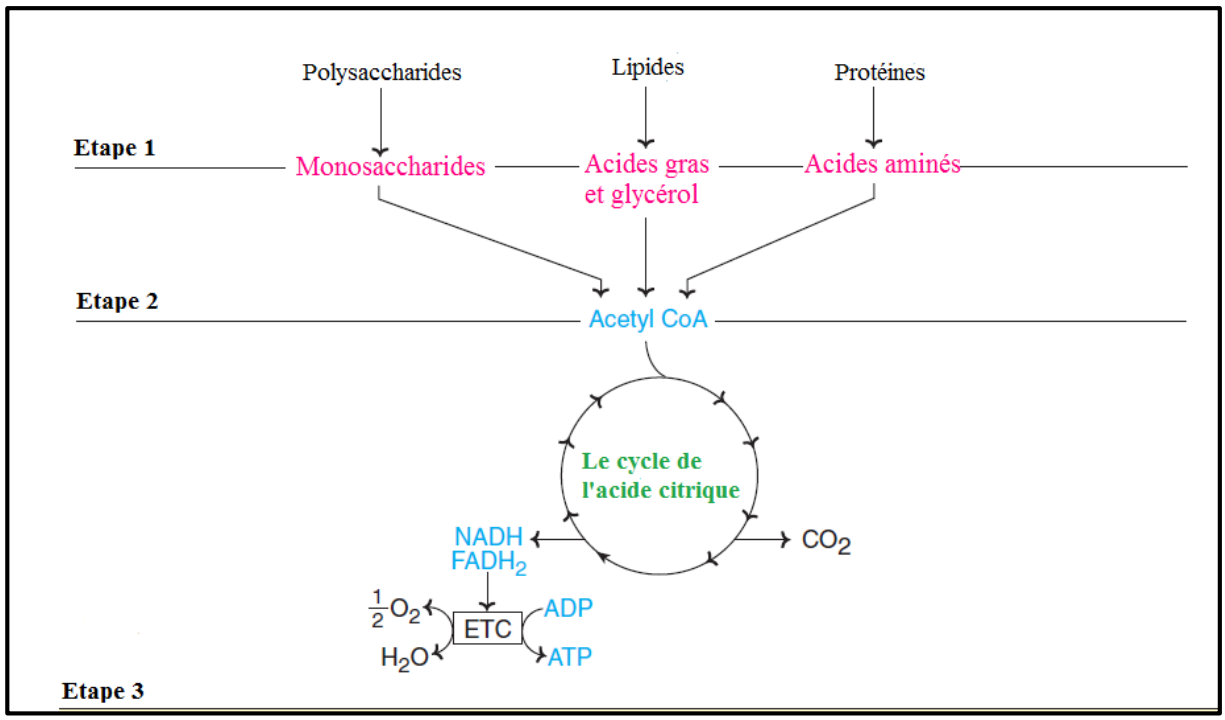

Figure 1: Steps of catabolism (ETC – Electron transport chain) (Satyanarayana and Chakrapani; 2013).

- **Anabolism :** The set of reactions that synthesize complex molecules from small molecules is known as anabolism. Anabolism is therefore a constructive process. Anabolic reactions require energy in the form of ATP. The synthesis of complex molecules builds tissues and organs. These complex molecules are necessary for cell growth, development, and differentiation. They increase muscle mass and mineralize bones (Lakna, 2017).

  Anabolism occurs in three steps :

  Step 1 : Involves production of precursors such as monosaccharides, nucleotides, amino acids, and isoprenoids.

  Step 2: The precursors produced in the first step are activated by ATP.

  Step 3: Activated precursors are assembled into complex molecules such as polysaccharides, nucleic acids, polypeptides and lipids (Lakna; 2017).

Table 1: Difference between anabolism and catabolism (Campbell and Farrell; 2009; Hanselius and Eldemark; 2010; Lakna; 2017).

Anabolism	**Catabolism**
The metabolic process that synthesizes complex molecules from simpler ones.	The metabolic process that breaks down complex molecules into smaller ones.
The constructive phase of metabolism.	The destructive phase of metabolism.
It is an endergonic reaction (requires energy (ATP)).	It is an exergonic reaction (releases energy (ATP)).
Estrogen, testosterone, growth hormones, insulin, etc. are involved.	Adrenaline, cortisol, glucagon, cytokines, etc. are involved.
It increases muscle mass. It builds, repairs and fills tissues.	It burns fat and calories. It uses stored food to generate energy.
Functional at rest or while sleeping.	Functional in bodily activities.
It is a reductive process.	It is an oxidative process.

Anabolic and catabolic pathways are not reversible and operate independently. Thus, metabolic pathways occur in specific cellular locations (mitochondria, microsomes, etc.) and are controlled by different regulatory signals (Satyanarayana and Chakrapani; 2013).

1.1. Organic reactions in metabolism

All common biological reactions can be classified into four groups:

- Group transfer reactions (transfer of an electrophile [acyl groups {RCOX}, phosphoryl groups {OP03X2-} and glycosyl groups] between nucleophiles [alcohols, amines, thiols, etc.]).
- Redox reaction.
- Eliminations {eliminate H2O, NH3, ROH or RNH2}, Isomerizations, Rearrangements.
- Formation or breaking of C-C bonds (condensation and cleavage reactions) (Gajera et al., 2008).

2. Metabolic pathways

2.1. Carbohydrate metabolism

2.1.1. Carbohydrates

Carbohydrates are the most abundant organic molecules in nature. They are primarily composed of the elements carbon, hydrogen, and oxygen. The name carbohydrate literally means « hydrate of carbon » Some carbohydrates have the empirical formula (CH2O)n where $n \leq 3$ which proves that these carbohydrates are indeed hydrate of carbon. However, there are several non-

carbohydrate compounds (e.g., acetic acid, $C_2H_4O_2$; lactic acid, $C_3H_6O_3$) that also appear as hydrate of carbon. Furthermore, some true carbohydrates (for example, rhamnohexose, $C_6H_{12}O_5$; deoxyribose, $C_5H_{10}O_4$) do not conform to the general formula. Therefore, carbohydrates cannot always be considered as hydrate of carbon. Carbohydrates can be defined as polyhydroxyaldehydes or ketones or compounds that produce them by hydrolysis. The term "sugar" applies to water-soluble carbohydrates and sweet taste (Satyanarayana and Chakrapani; 2013).

2.1.2. Functions of carbohydrates

Carbohydrates play a multitude of roles in living organisms:

* Carbohydrates are the most abundant dietary source of energy for all organisms.
* Carbohydrates are the precursors of many organic compounds (fats, amino acids).
* Carbohydrates (in the form of glycoproteins and glycolipids) participate in the structure of the cell membrane and in cellular functions such as cell growth, adhesion and fertilization.
* Carbohydrates provide cells with external protection. Cellulose supports (literally) the entire plant kingdom.
* Carbohydrates also serve as a form of energy storage (glycogen) to fulfill the body's immediate energy needs (Satyanarayana and Chakrapani, 2013; Mougios, 2020).

2.1.3. Main carbohydrate metabolic pathways

2.1.3.1. Glycolysis

A. Definition

Glycolysis is often called the Embden-Meyerhof pathway. It is the main pathway of glucose metabolism and occurs in the cytosol of all cells. This pathway can use O_2 if it is available "aerobic" and it can also function in the absence of O_2 "anaerobic". In the glycolysis pathway, glucose is split into two 3-carbon pyruvate molecules under aerobic conditions; or lactate under anaerobic conditions, with the production of a small amount of energy (Gilbert, 2000; Solomon et al., 2004; Murray et al., 2009; Vasudevan et al., 2011; Satyanarayana and Chakrapani, 2013).

B. Steps of glycolysis

- **Phosphorylation of glucose**

Glucose is phosphorylated to glucose-6-phosphate by hexokinase or glucokinase in an irreversible reaction that depends on ATP and Mg^{2+}. Hexokinase is present in almost all tissues. It catalyzes the phosphorylation of various hexoses (fructose, mannose, etc.), and has a low Km for substrates (approximately 0.1 mM). Glucokinase, present in the liver, catalyzes the

phosphorylation of glucose only, has a high Km for glucose (10 mM) (Berg et al., 2002; Vasudevan et al., 2011; Satyanarayana and Chakrapani, 2013).

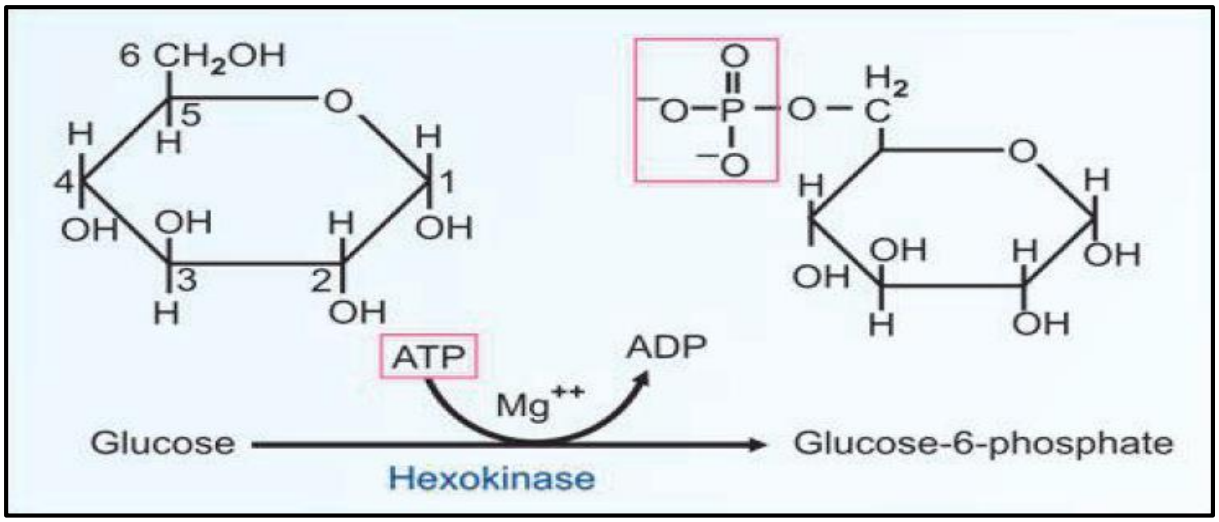

Figure 2: First reaction of glycolysis (Vasudevan et al., 2011).

- **Isomerization of glucose-6-phosphate**

Glucose-6-phosphate is isomerized into fructose-6-phosphate by the enzyme phosphohexose isomerase in the presence of Mg^{2+}. This is a reversible reaction (Solomon et al., 2004; Satyanarayana and Chakrapani, 2013).

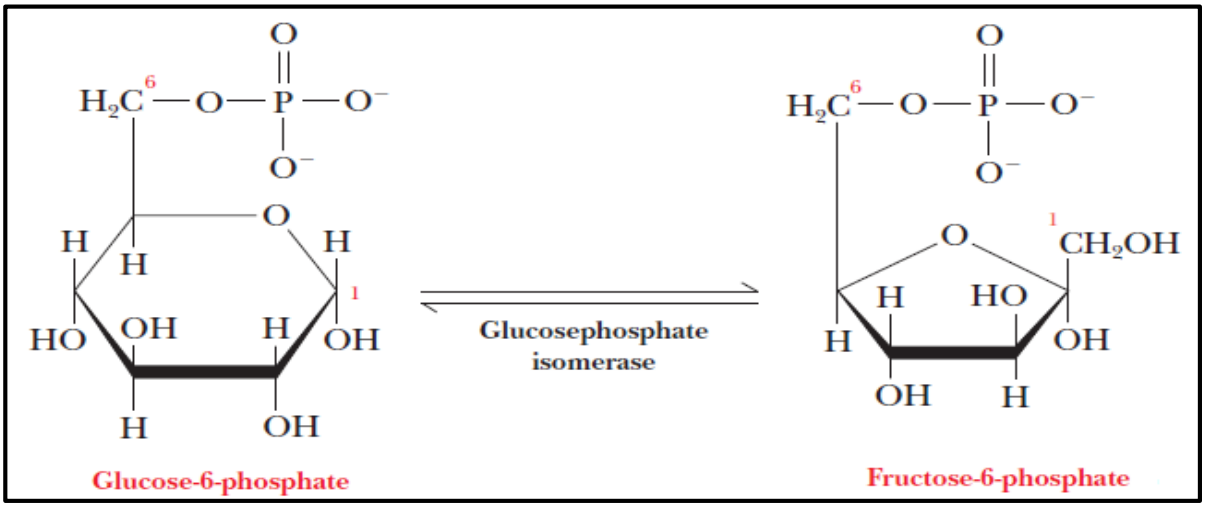

Figure 3: Second reaction of glycolysis (Campbell and Farrell, 2009).

- **Phosphorylation of fructose-6-phosphate**

Fructose-6-phosphate is phosphorylated to fructose-1,6-bisphosphate by phosphofructokinase (PFK). This is an irreversible and regulatory step in glycolysis. Note that at this stage, the oxidation of glucose does not produce any useful energy, but rather there is a use of 2 ATP

molecules for two phosphorylations (-2 ATP) (Berg et al., 2002; Solomon et al., 2004; Satyanarayana and Chakrapani, 2013).

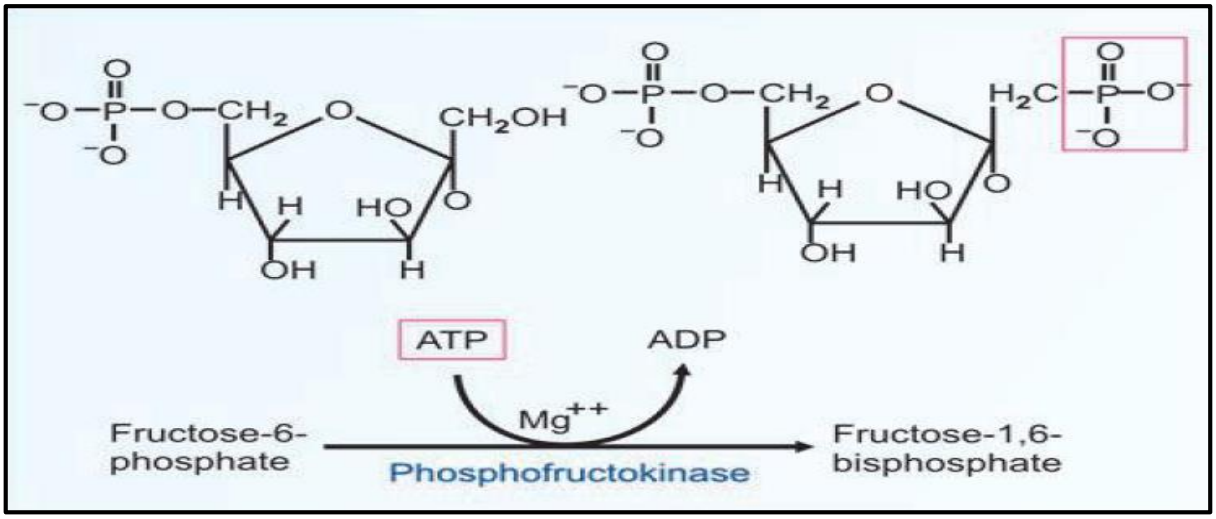

Figure 4: Third reaction of glycolysis (Vasudevan et al., 2011).

- **Cleavage of fructose-1,6-bisphosphate**

Six-carbon fructose-1,6-bisphosphate is cleaved into two three-carbon compounds, glyceraldehyde-3-phosphate (GAP) and dihydroxyacetone phosphate (DHAP) by the enzyme aldolase (fructose-1,6-bisphosphate aldolase) (Berg et al., 2002; Solomon et al., 2004; Satyanarayana and Chakrapani, 2013).

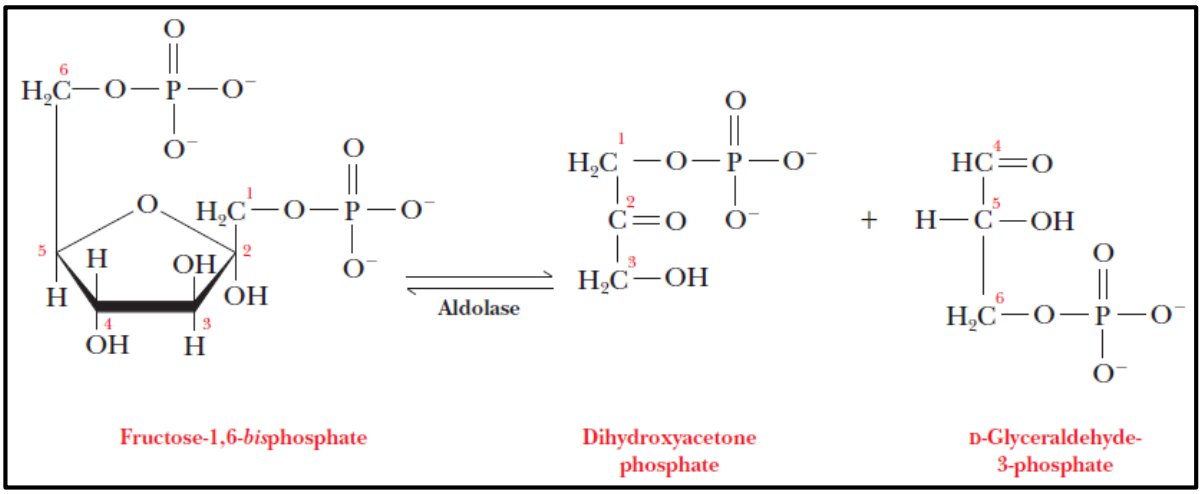

Figure 5: Fourth reaction of glycolysis (Campbell and Farrell, 2009).

- **Isomerization of dihydroxyacetone phosphate**

The enzyme triosephosphate isomerase catalyzes the reversible interconversion of glyceraldehyde-3-phosphate and dihydroxyacetone phosphate. Thus, two molecules of glyceraldehyde-3-phosphate are obtained from one molecule of glucose(Berg et al., 2002; Satyanarayana and Chakrapani, 2013).

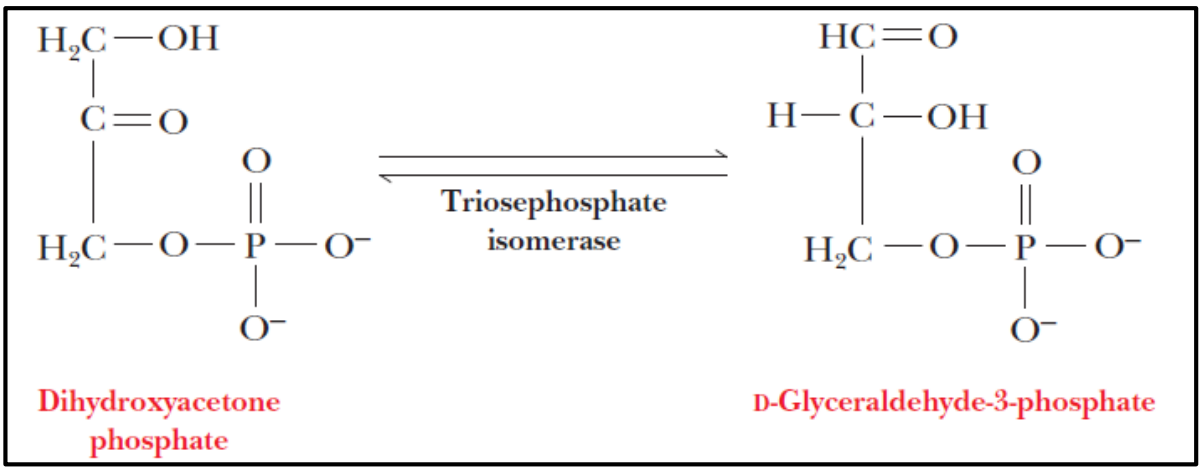

Figure 6: Fifth reaction of glycolysis (Campbell and Farrell, 2009).

- **Oxidation (and phosphorylation) of glyceraldehyde-3-phosphate**

Glycerlaldehyde-3-phosphate dehydrogenase converts glyceraldehyde-3-phosphate to 1,3-bisphosphoglycerate. This step is important because it is involved in the formation of NADH, H+, and a high-energy compound, 1,3-bisphosphoglycerate (Berg et al., 2002; Satyanarayana and Chakrapani, 2013).

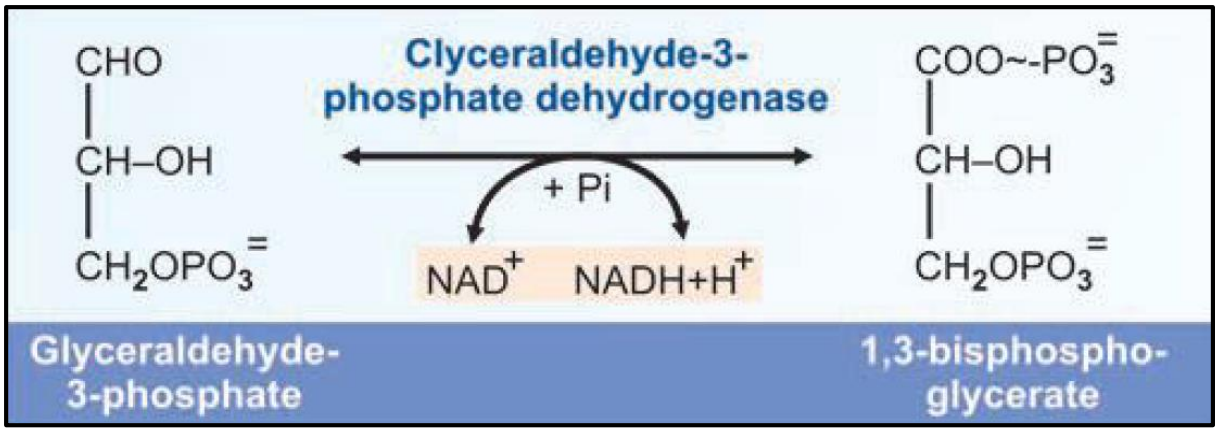

Figure 7: Sixth reaction of glycolysis (Vasudevan et al., 2011).

- **Transfer of a phosphate group from 1,3-bisphosphoglycerate to ADP**

The phosphoglycerate kinase enzyme acts on 1,3-bisphosphoglycerate, leading to ATP synthesis and the formation of 3-phosphoglycerate. This step is a good example of substrate-level phosphorylation, since ATP is synthesized from the substrate without the involvement of the electron transport chain. The phosphoglycerate kinase reaction is reversible, a rare example among kinase reactions (Satyanarayana and Chakrapani, 2013).

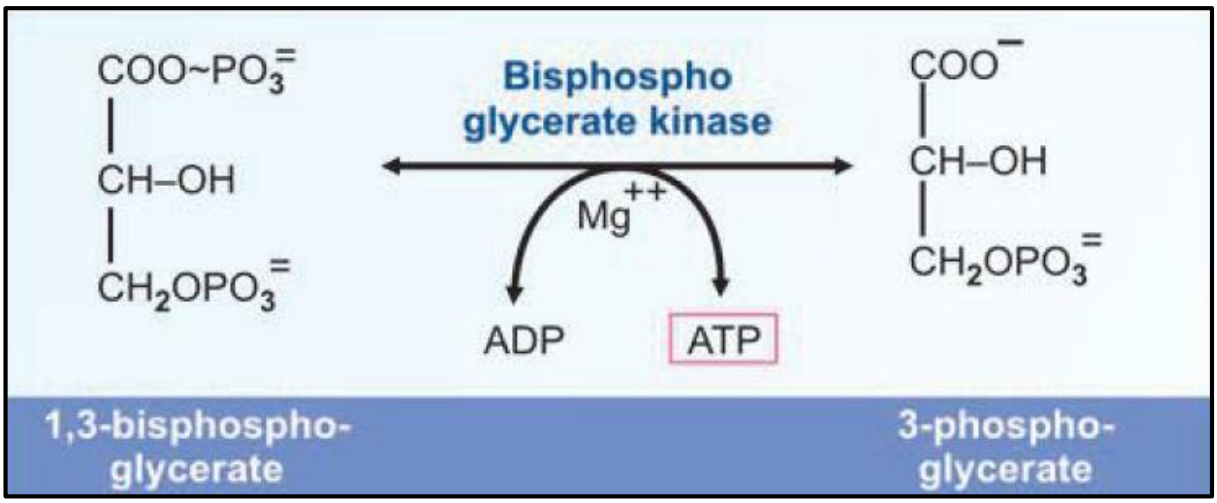

Figure 8: Seventh reaction of glycolysis (Vasudevan et al., 2011).

- **Isomerization of 3-phosphoglycerate**

3-phosphoglycerate is converted to 2-phosphoglycerate by phosphoglycerate mutase. Mg^{2+} is essential for this reaction (Berg et al., 2002; Nelson and Cox, 2005; Satyanarayana and Chakrapani, 2013).

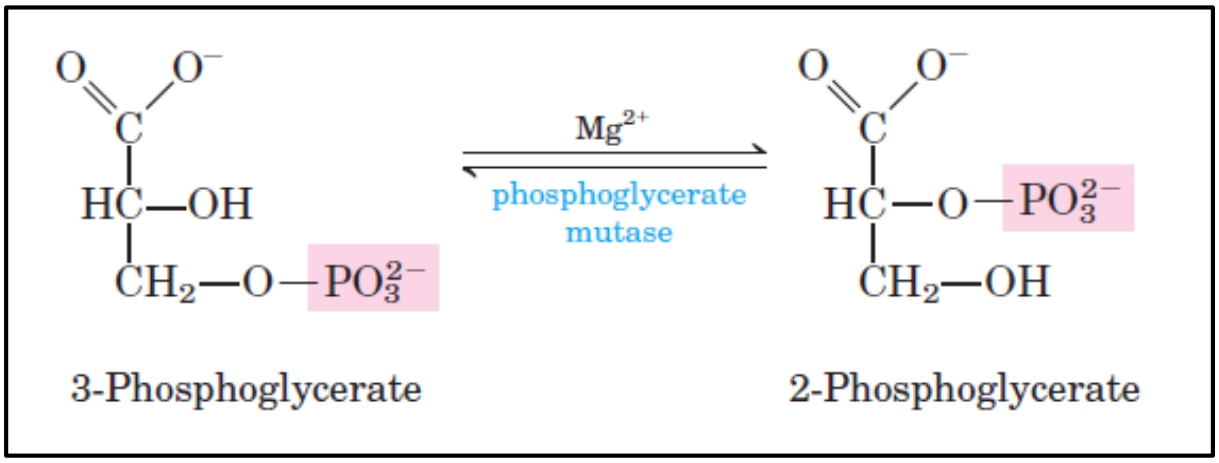

Figure 9: Eighth reaction of glycolysis (Nelson and Cox, 2005).

- **Dehydration of 2-phosphoglycerate**

This step is catalyzed by enolase and involves dehydration, forming phosphoenolpyruvate. Enolase requires Mg^{2+} or Mn^{2+} (Murray et al., 2009; Satyanarayana and Chakrapani, 2013).

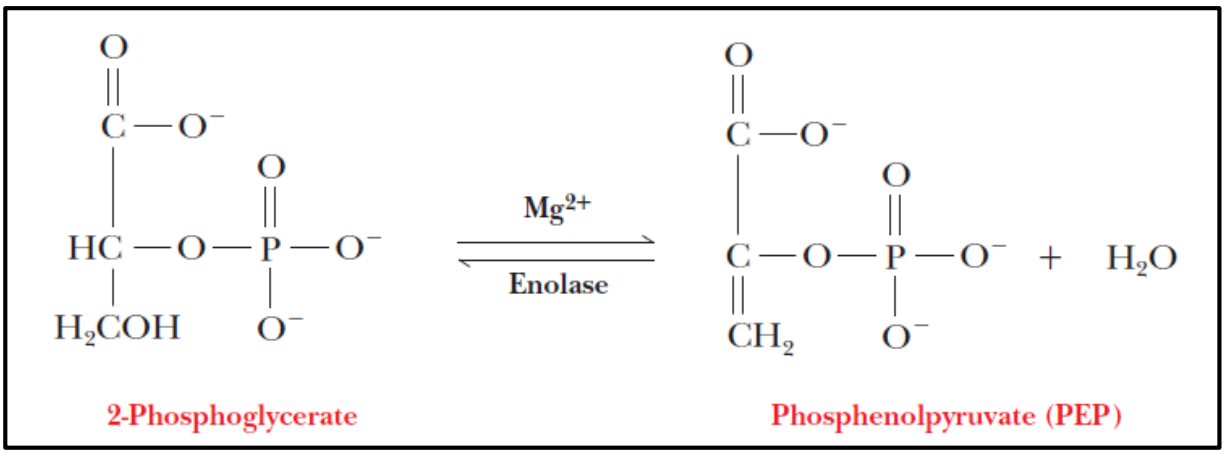

Figure 10: Ninth reaction of glycolysis (Campbell and Farrell, 2009).

- **Phosphate transfer from phosphoenolpyruvate to ADP**

The enzyme pyruvate kinase catalyzes the high-energy transfer of phosphate from phosphoenolpyruvate to ADP, leading to ATP formation. This step is also a substrate-level phosphorylation. This reaction is irreversible (Satyanarayana and Chakrapani, 2013).

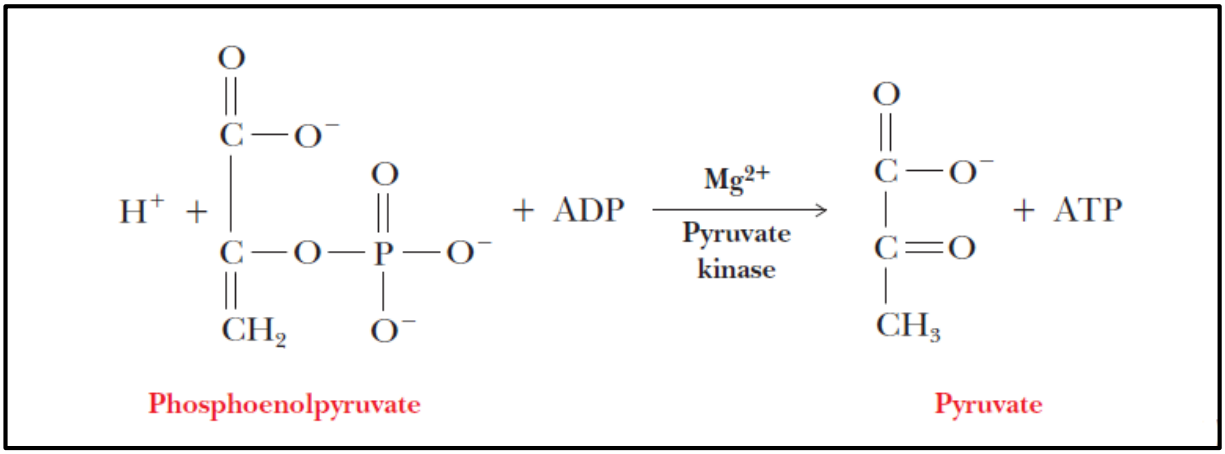

Figure 11: Tenth reaction of glycolysis (Campbell and Farrell, 2009).

C. Energy balance

Table 2: Products formed during the conversion of one glucose molecule into two pyruvate molecules in glycolysis (Meisenberg and Simmons, 2017).

Enzyme	**Product (molecules)**
Hexokinase	−1 ATP
Phosphofructokinase	−1 ATP
Glyceraldehyde-3-phosphate dehydrogenase	+2 NADH
Phosphoglycerate kinase	+2 ATP
Pyruvate kinase	+2 ATP

Overall, glycolysis reactions produce a net yield of two molecules of ATP and two molecules of NADH for each molecule of glucose (Meisenberg and Simmons, 2017).

$$\textbf{Glucose + 2P}_i\textbf{ + 2ADP + 2NAD}^+ \rightarrow \textbf{2Pyruvate + 2ATP + 2NADH + 2H}^+\textbf{ + 2H}_2\textbf{O}$$

D. Fates of pyruvate

- **Decarboxylation of pyruvate to Acetyl-CoA**

Under aerobic conditions, pyruvate is oxidized in the mitochondria. It diffuses through the pores of the outer mitochondrial membrane and is transported across the inner mitochondrial membrane by a specialized transporter. In the mitochondrial matrix, it is decarboxylated by oxidation to acetyl-CoA (Meisenberg and Simmons, 2017) :

$$\textbf{Pyruvate + NAD + CoA-SH} \rightarrow \textbf{Acetyl-CoA + NADH + CO}_2$$

- **Reduction of pyruvate to lactate**

The second pathway for pyruvate is its reduction to lactate via lactic acid fermentation. The reduction of pyruvate by NADH is catalyzed by lactate dehydrogenase (Berg et al., 2002; Nelson and Cox, 2005; Campbell and Farrell, 2009).

$$\textbf{Pyruvate + NADH + H}^+ \rightarrow \textbf{Lactate + NAD}^+$$

- **Transformation of pyruvate into ethanol**

Two other reactions linked to the glycolytic pathway lead to the production of ethanol by alcoholic fermentation.

The first step is the decarboxylation of pyruvate. This reaction is catalyzed by pyruvate decarboxylase (Berg et al., 2002; Campbell and Farrell, 2009).

$$\textbf{Pyruvate} \rightarrow \textbf{Acetaldehyde}$$

The second step is the reduction of acetaldehyde to ethanol by NADH, in a reaction catalyzed by alcohol dehydrogenase. This process regenerates NAD^+ (Berg et al., 2002; Campbell and Farrell, 2009).

$$\textbf{Acetaldehyde + NADH} \rightarrow \textbf{Ethanol + NAD}^+$$

E. Importance of glycolysis

1. It is the only pathway that occurs in all cells of the body.
2. Glycolysis is the only source of energy for erythrocytes.
3. During intense exercise, when muscle tissue lacks oxygen, anaerobic glycolysis is the main source of energy for the muscles.
4. The glycolytic pathway can be considered as the preliminary step before complete oxidation.

5. The glycolytic pathway provides carbon skeletons for the synthesis of non-essential amino acids as well as glycerol, a part of fats.

6. Most reactions of glycolysis are reversible, which are also used for gluconeogenesis (Vasudevan et al., 2011).

2.1.3.2. Krebs cycle

A. Definition

The Krebs cycle (Citric Acid Cycle, Tricarboxylic Acid Cycle) is a series of reactions in the mitochondria that oxidizes the acetyl group of acetyl-CoA and reduces coenzymes that are reoxidized by the electron transport chain, linked to the formation of ATP (Murray et al., 2009).

The Krebs cycle is amphibolic, meaning that it participates in both anabolism and catabolism. Its metabolites are transported to the cytosol where they provide building blocks for macromolecules synthesis (Gajera et al., 2008; Campbell and Farrell, 2009; Martínez-Reyes and Chandel, 2020).

The Krebs cycle is an aerobic pathway and is considered the final common pathway for the oxidation of carbohydrates, lipids, and proteins because glucose, fatty acids, and most amino acids are metabolized to acetyl-CoA or cycle intermediates. It also plays a central role in gluconeogenesis, lipogenesis, and amino acid interconversion (Campbell and Farrell, 2009; Murray et al., 2009).

B. Steps of the Krebs cycle

- **Preparatory step**

Under aerobic conditions, pyruvate is transported into the mitochondria via a specific transporter. There, the pyruvate dehydrogenase complex converts pyruvate into carbon dioxide and acetyl-CoA. The resulting acetyl-CoA then enters the Citric Acid Cycle (Berg et al., 2002; Campbell and Farrell, 2009).

- **Citrate formation**

The Krebs cycle begins with the condensation of oxaloacetate and acetyl CoA. Oxaloacetate reacts with acetyl CoA and H_2O to produce citrate and CoA. This irreversible reaction is catalyzed by citrate synthase (Berg et al., 2002; Murray et al., 2009; Meisenberg and Simmons, 2017).

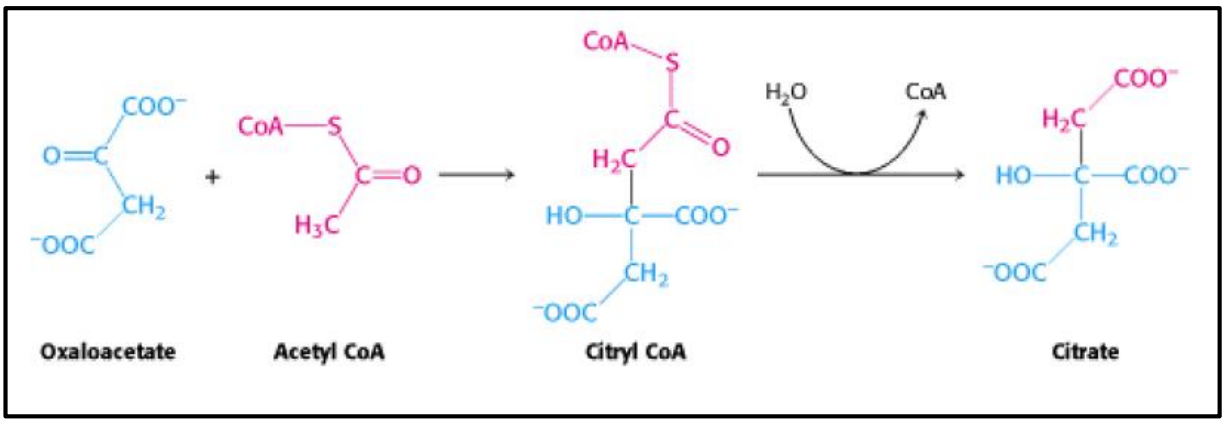

Figure 12: Condensation of acetyl-CoA and oxaloacetate into citrate (Berg et al., 2002).

- **Isomerization of citrate**

Citrate is isomerized to isocitrate by aconitase (aconitate hydratase). The reaction occurs in two steps: dehydration to cis-aconitate and rehydration to isocitrate (Murray et al., 2009; Meisenberg and Simmons, 2017).

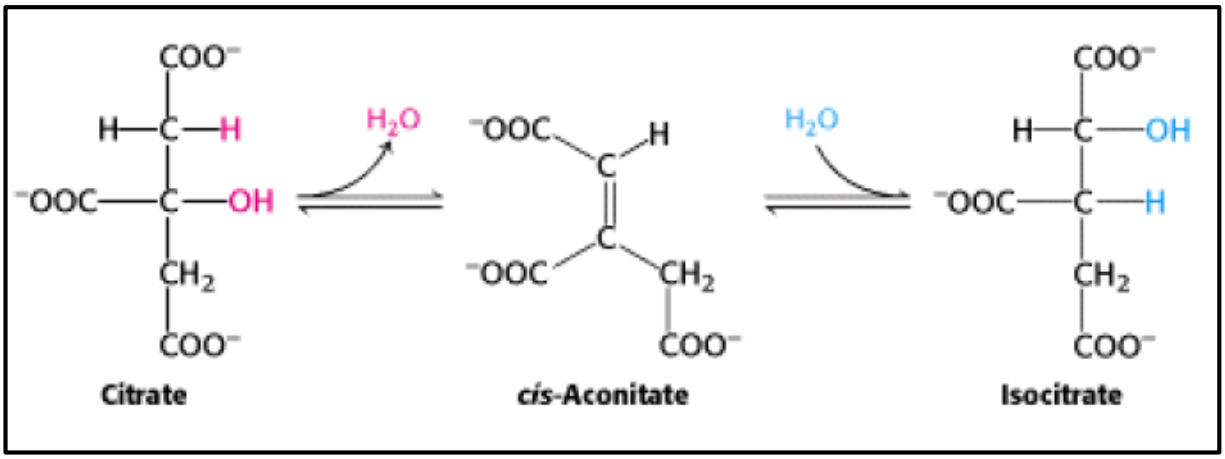

Figure 13: Isomerization of citrate (Berg et al., 2002).

- **Oxidative decarboxylation of isocitrate**

Isocitrate undergoes oxidative decarboxylation to initially form oxalosuccinate, which remains bound to the enzyme and undergoes decarboxylation to α-ketoglutarate. The reaction is catalyzed by isocitrate dehydrogenase. Isocitrate dehydrogenase of the Krebs cycle is an NAD-bound enzyme (Berg et al., 2002; Murray et al., 2009; Meisenberg and Simmons, 2017).

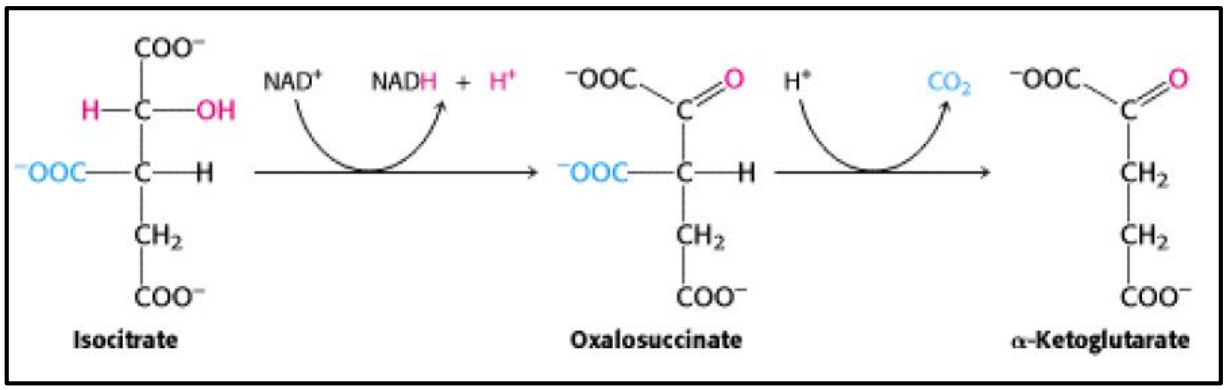

Figure 14: Oxidative decarboxylation of isocitrate (Berg et al., 2002).

- **Oxidative decarboxylation of α-ketoglutarate**

The conversion of isocitrate to α-ketoglutarate is followed by a second oxidative decarboxylation reaction, the formation of succinyl CoA from α-ketoglutarate. This reaction is catalyzed by α-ketoglutarate dehydrogenase (Berg et al., 2002; Meisenberg and Simmons, 2017).

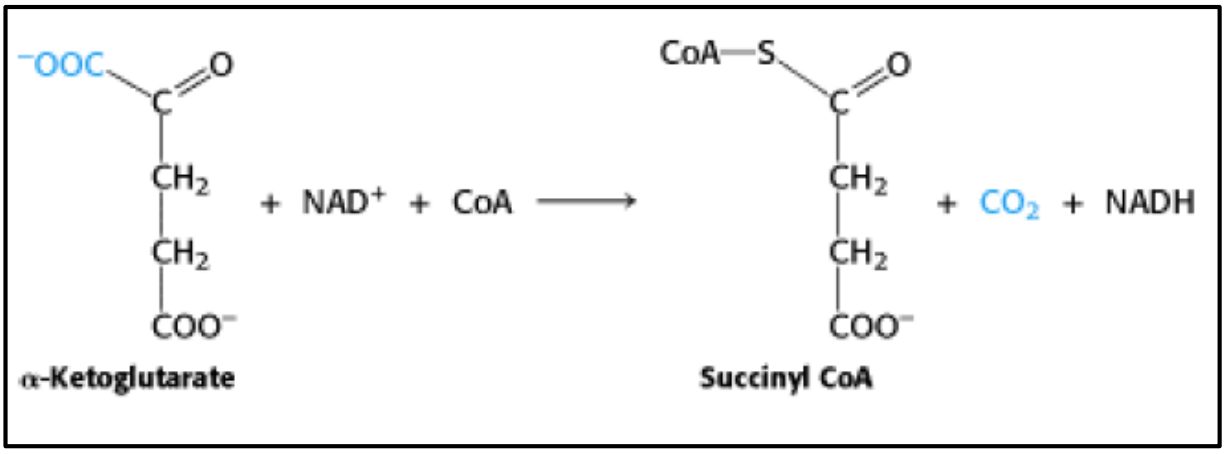

Figure 15: Oxidative decarboxylation of α-ketoglutarate (Berg et al., 2002).

- **Succinate generation**

Succinyl-CoA is converted to succinate by succinate thiokinase (succinyl-CoA synthetase). Two forms of this enzyme exist in human tissues, which link this reaction to the synthesis of ATP or GTP, respectively. The ATP-generating enzyme predominates in the brain and heart, while the GTP-generating enzyme predominates in the liver (Murray et al., 2009; Meisenberg and Simmons, 2017).

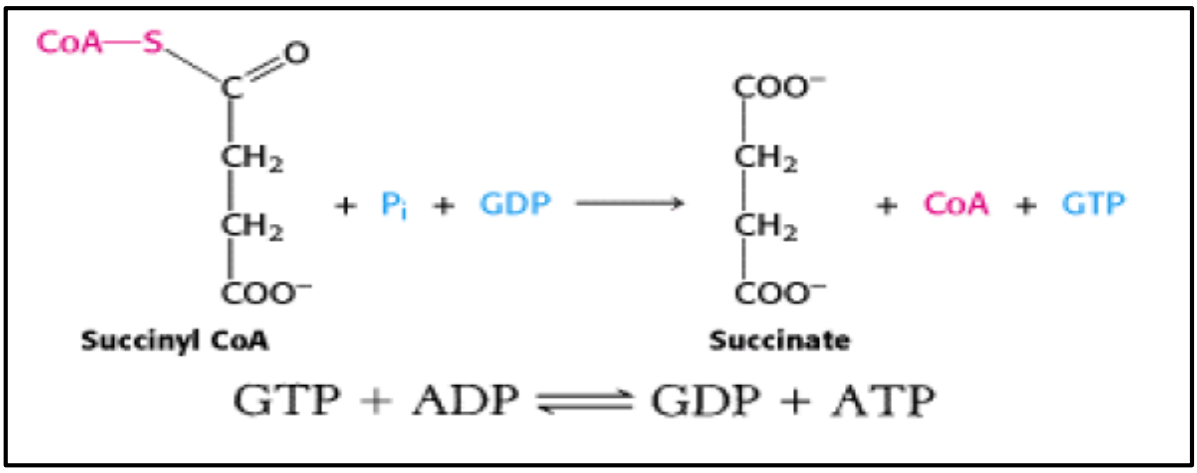

Figure 16: Phosphorylation of succiny-CoA (Berg et al., 2002).

- **Fumarate formation**

Succinate is dehydrogenated to fumarate by succinate dehydrogenase. The hydrogen acceptor is FAD rather than NAD+ (Berg et al., 2002; Murray et al., 2009).

- **Malate formation**

Fumarate is hydrated to malate by fumarase (fumarate hydratase) (Meisenberg and Simmons, 2017).

- **Oxaloacetate regeneration**

Malate is oxidized to oxaloacetate in the NAD^+-dependent malate dehydrogenase reaction (Meisenberg and Simmons, 2017).

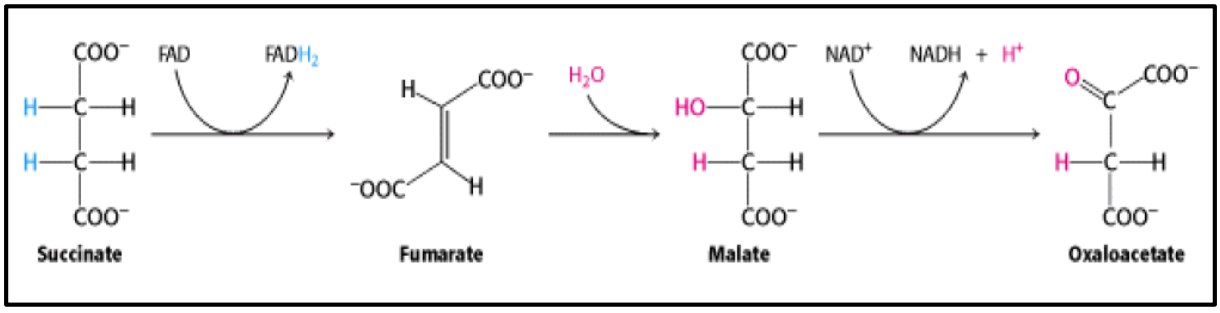

Figure 17: Oxaloacetate regeneration (Berg et al., 2002).

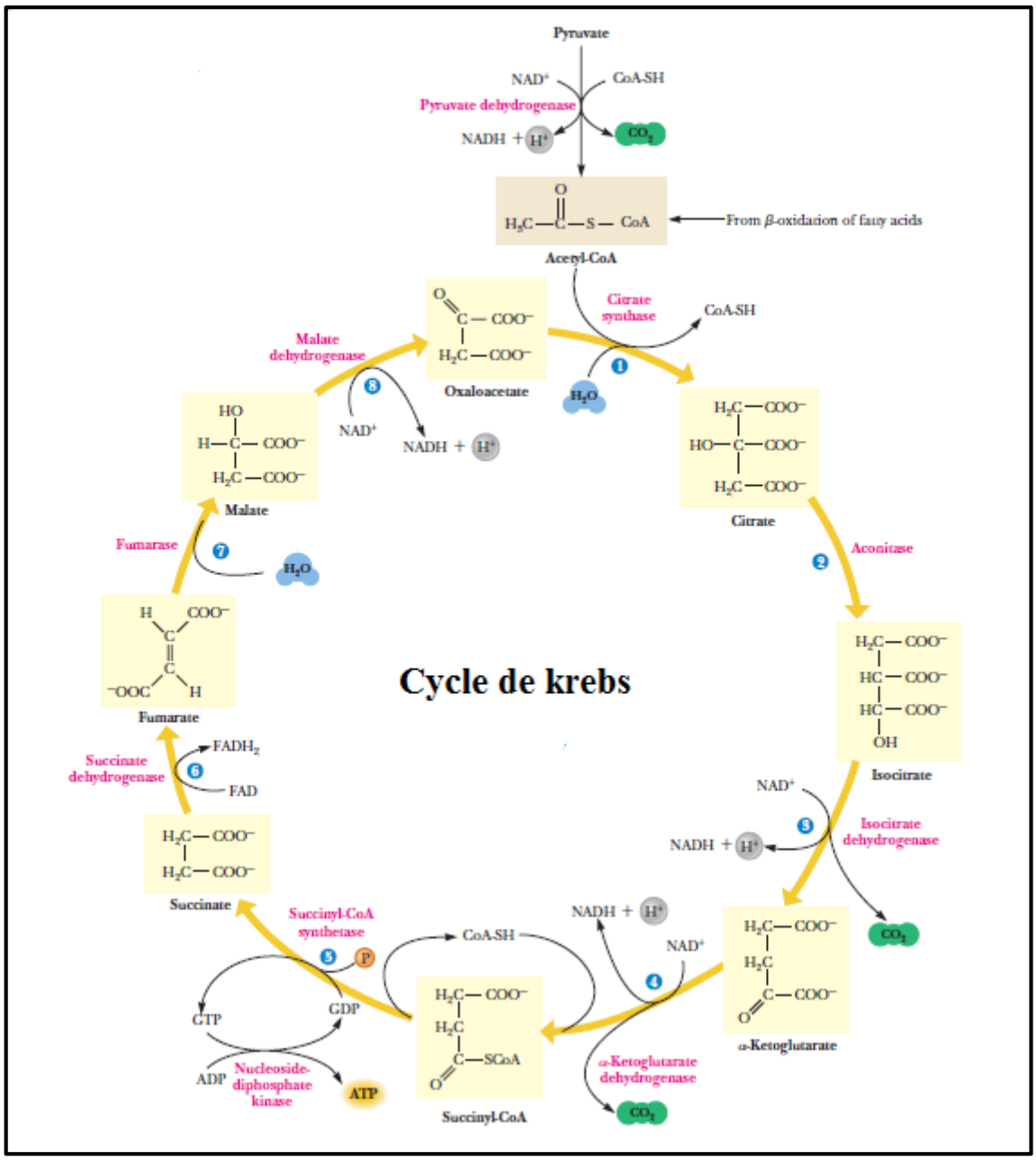

Figure 18: Krebs cycle (Campbell and Farrell, 2009).

C. Energy balance

-Three NADH molecules are generated in each cycle, and each yields 3 ATP molecules, giving a total of 3 × 3 = 9 ATP.

-$FADH_2$ is produced, yielding 2 ATP molecules.

- One GTP (equivalent to one ATP) is also formed.

*Thus, each turn of the cycle produces a total of 12 ATP molecules (Meisenberg and Simmons, 2017).

2.1.3.3. Gluconeogenesis

A. Definition

Gluconogenesis is the synthesis of glucose from non-carbohydrate precursors. Lactate, glycerol, certain glucogenic amino acids (alanine and glutamine), pyruvate, and propionate are the main substrates of gluconeogenesis. Gluconeogenesis occurs primarily in the liver and, to lesser extent, in the renal matrix. The pathway is partly mitochondrial and partly cytoplasmic (Berg et al., 2002; Vasudevan et al., 2011; Satyanarayana and Chakrapani, 2013; Shah and Wondisford, 2023; Sahoo et al., 2023).

B. Key enzymes

-Pyruvate carboxylase

-Phosphoenolpyruvate carboxykinase

-Fructose-1-6-bisphosphatase

-Glucose-6-phosphatase (Vasudevan et al., 2011).

C. Steps of gluconeogenesis

Gluconogenesis involves several glycolytic enzymes, but it is not a reversal of glycolysis (Berg et al., 2002; Vasudevan et al., 2011). The irreversible reactions of glycolysis (the first, third, and tenth) are bypassed by the four key enzymes of gluconeogenesis (Vasudevan et al., 2011; Mougios, 2020).

- **Conversion of pyruvate to oxaloacetate**

Pyruvate in the cytoplasm enters the mitochondria, where it is carboxylated to oxaloacetate by the mitochondrial enzyme pyruvate carboxylase. This reaction requires biotin and ATP as coenzymes. Because most subsequent gluconeogenic reactions occur in the cytosol, oxaloacetate is reduced to malate, which can be transported out of the mitochondria via the malate–aspartate shuttle. Once in the cytosol, malate is re-oxidized to oxaloacetate by malate dehydrogenase, an enzyme present in both the mitochondrial matrix and the cytoplasm (Vasudevan et al., 2011; Satyanarayana and Chakrapani, 2013; Mougios, 2020).

- **Conversion of oxaloacetate to phosphoenolpyruvate**

In the cytosol, phosphoenolpyruvate carboxykinase (PEPCK) converts oxaloacetate into phosphoenolpyruvate, using GTP or ITP as the energy source and releasing CO_2. The overall conversion of pyruvate to phosphoenolpyruvate requires two ATP equivalents, in contrast to glycolysis, where the reverse step yields a single ATP. Phosphoenolpyruvate then proceeds through several reactions catalyzed by glycolytic enzymes to form fructose-1,6-bisphosphate

(corresponding to glycolysis steps 4, 5, 6, 7, 8). All of these steps are reversible (Vasudevan et al., 2011; Satyanarayana and Chakrapani, 2013).

- **Conversion of fructose 1,6-bisphosphate to fructose-6-phosphate**

  Phosphoenolpyruvate undergoes reverse glycolysis until fructose-1,6-bisphosphate is produced. The enzyme fructose-1,6-bisphosphatase converts fructose-1,6-bisphosphate to fructose-6-phosphate (Vasudevan et al., 2011; Satyanarayana and Chakrapani, 2013).

- **Conversion of fructose-6-phosphate to glucose-6-phosphate**

  Phosphohexose isomerase converts fructose-6-phosphate to glucose-6-phosphate (Melkonian et al., 2023).

- **Conversion of glucose-6-phosphate to glucose**

  Glucose-6-phosphate is hydrolyzed to glucose by glucose-6-phosphatase (Vasudevan et al., 2011; Satyanarayana and Chakrapani, 2013).

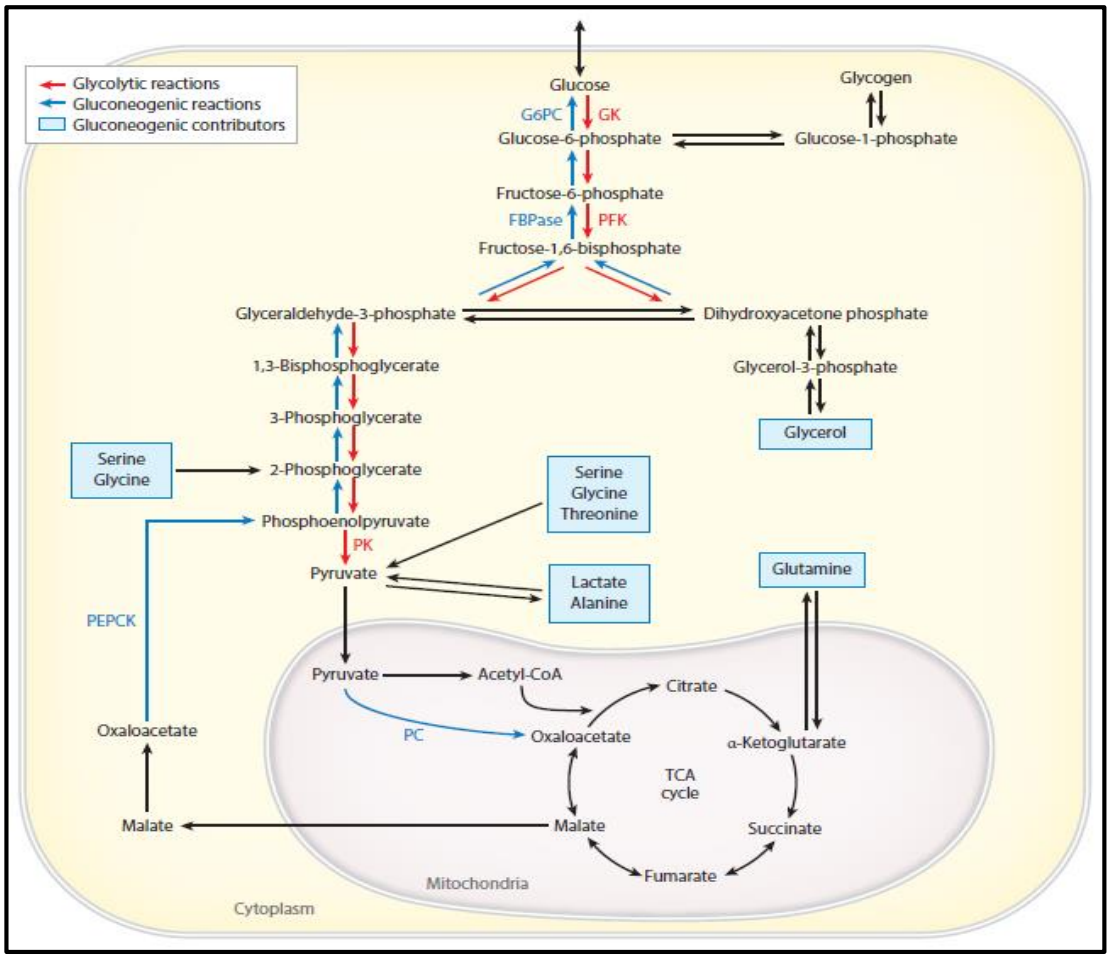

Figure 19: Gluconeogenesis (Shah and Wondisford, 2023).

D. Energy balance

The reactions catalyzed by pyruvate carboxylase, phosphoenolpyruvate carboxykinase and phosphoglycerate kinase each require one ATP; Consequently, a total of 3 ATP are consumed for the conversion of a single pyruvate molecule into half a molecule of glucose, meaning that 6 ATP are required to produce one full molecule of glucose (Vasudevan et al., 2011).

$$\mathbf{2\ Pyruvate + 4ATP + 2GTP + 2NADH + 2H^{+} + 6H_2O \rightarrow Glucose + 2NAD^{+} + 4ADP + 2GDP + 6Pi + 6H^{+}}$$

2.1.3.4. Glycogenesis

A. Definition

Glycogen is a glucose polymer that serves as the primary storage form of glucose in the cytoplasm of the liver and muscle tissue, and in small amounts in various other organs such as the brain, kidneys, and heart (Roach et al., 2012; Kanungo et al., 2018; Neoh et al., 2024).

Glycogenesis is an anabolic pathway of glucose (from glucose to glycogen) (Nelson and Cox, 2005). It takes place in the cytosol of myocytes and hepatocytes and requires ATP, UTP and glucose (Satyanarayana and Chakrapani, 2013; Bhat and Kumar, 2017; Pacheco-Gómez et al., 2021).

B. Steps of glycogenesis

- **Phosphorylation of glucose**

The starting point of glycogen synthesis is the phosphorylation of glucose to glucose-6-phosphate in an irreversible enzymatic reaction catalyzed by hexokinase (in muscle) and glucokinase (in the liver) (Nelson and Cox, 2005; Adeva-Andany et al., 2016; Bhat and Kumar, 2017):

Glucose + ATP → Glucose-6-phosphate+ADP

- **Glucose isomerization**

Glucose-6-phosphate undergoes isomerization to glucose-1-phosphate by phosphoglucomutase (Nelson and Cox, 2005; Adeva-Andany et al., 2016; Pacheco-Gómez et al., 2021).

Glucose 6-phosphate ↔Glucose-1-phosphate

- **Formation of uridine diphosphate-glucose**

The third step involves the conversion of glucose-1-phosphate into the active nucleotide uridine diphosphate glucose (UDP-glucose), the immediate precursor of glycogen synthesis by uridine triphosphate (UTP) in an enzymatic reaction catalyzed by glucose-1-phosphate

uridylyltransferase (or UDP-glucose pyrophosphorylase) (Nelson and Cox, 2005; Bhat and Kumar, 2017).

Glucose-1-phosphate +UTP → UDP-glucose + PPi

- **Initiation of glycogen synthesis**

Glycogen biogenesis from UDP-glucose involves two enzymes : glycogen synthase and amylo-α(1,4→1,6)-glucosyltransferase. Glycogen synthase catalyzes the transfer of the glycosyl group from UDP-glucose to the non-reducing ends of a pre-existing tetrasaccharide composed of four glucosyl residues linked in α(1,4). The first residue of this tetrasaccharide is linked to a specific amino acid, tyrosine, at position 194 in a "primer" protein called glycogenin, which has Mg^{2+}-dependent autocatalytic activity. The hydroxyl group of this tyrosine is the site to which the initial glucose unit is attached (Bhat and Kumar, 2017).

- **Glycogen chain elongation**

Further elongation of the linear chain is catalyzed by glycogen synthase by transferring glucose from UDP-glucose to the growing glycogen chain via an α(1,4)-glycosidic linkage (Bhat and Kumar, 2017).

- **Glycogen branching**

Amylo-α(1,4→1,6)-glucosyltransferase, also known as the branching enzyme, catalyzes the formation of glycogen branches by cleaving α(1,4) linkages in a linear chain and attaching the resulting fragment to the C6 position of a glucose residue. This process creates a branch of approximately seven glucose units connected via an α(1,6) linkage to the main glycogen chain (Bhat and Kumar, 2017).

2.1.3.5. Glycogenolysis

A. Definition

Glycogenolysis is the breakdown of glycogen stored in the liver and muscles (Satyanarayana and Chakrapani, 2013). During this process, multiple glucose residues can be released simultaneously, one from each branch end. This feature allows the organism to rapidly increase glucose availability, efficiently meeting short-term energy demands (Campbell and Farrell, 2009).

B. Steps of glycogenolysis

- **Action of glycogen phosphorylase**

The α-1,4-glycosidic bonds (non-reducing ends) are sequentially cleaved by the enzyme glycogen phosphorylase to produce glucose-1-phosphate. This process, called phosphorolysis,

continues until four glucose residues remain on each side of the branch point (α-1,6 glycosidic bond). The glycogen thus formed is known as limit dextrin, which can no longer be degraded by phosphorylase (Murray et al., 2009; Satyanarayana and Chakrapani, 2013; Meisenberg and Simmons, 2017).

- **Conversion of glucose-1-phosphate to glucose-6-phosphate**

  Glucose-1-phosphate, the final product of the glycogen phosphorylase reaction, is converted to glucose-6-phosphate by phosphoglucomutase, which catalyzes the reversible reaction (Nelson and Cox, 2005).

- **Glucose formation**

  The fate of glucose-6-phosphate depends on the tissue. The liver, kidneys, and intestines contain the enzyme glucose-6-phosphatase, which converts glucose-6-phosphate to glucose. This enzyme is absent in muscles and the brain, so free glucose cannot be produced from glucose-6-phosphate in these tissues (Satyanarayana and Chakrapani, 2013).

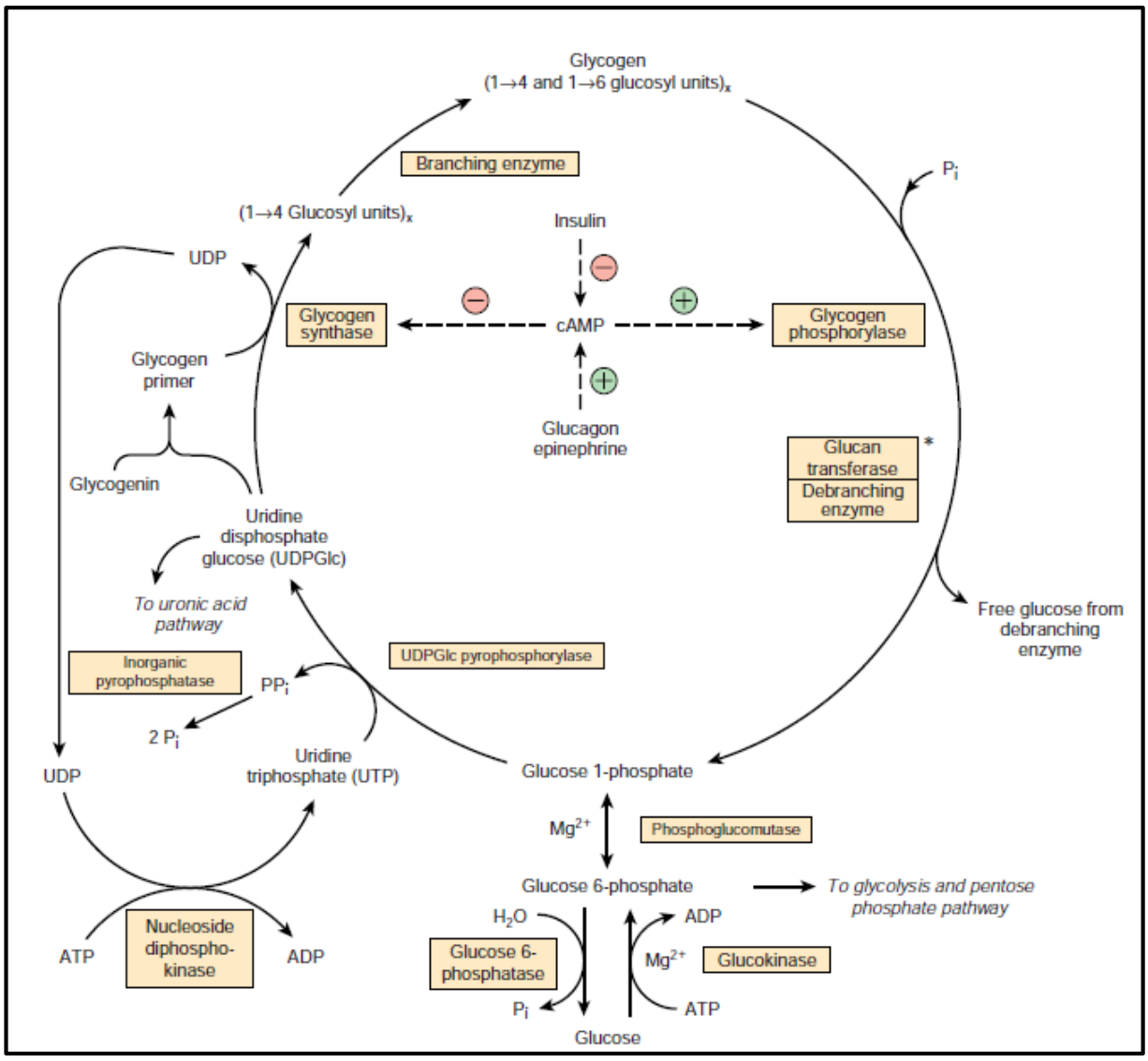

Figure 20: Glycogenesis and glycogenolysis in the liver (⊕, stimulation ; ⊖, inhibition) (Murray et al., 2009).

2.1.3.6. Pentose phosphate pathway

A. Definition

This process takes place in the cytosol and is divided into two phases: the oxidative phase and the non-oxidative phase (Pacheco-Gómez et al., 2021). Tissues such as the liver, adipose tissue, adrenal gland, erythrocytes, testes, and lactating mammary gland are highly active in the pentose phosphate pathway (Solomon et al., 2004; Satyanarayana and Chakrapani, 2013).

During the oxidative phase, glucose-6-phosphate is oxidized, generating two molecules of NADPH and one molecule of pentose phosphate, with the release of one molecule of CO_2. During the non-oxidative phase, pentose phosphate is converted into glycolysis intermediates (Vasudevan et al., 2011).

The main functions of this pathway are :

- To generate reducing equivalents, in the form of NADPH, for reductive biosynthetic reactions within cells.

- To provide the cell with ribose-5-phosphate (R5P) for the synthesis of nucleotides and nucleic acids.

- The pentose phosphate pathway plays a role in metabolism by degrading pentose sugars resulting from the digestion of nucleic acids and by reorganizing the carbon skeletons of dietary carbohydrates into glycolytic and gluconeogenic intermediates (Solomon et al., 2004).

B. Steps of pentose phosphate pathway

- **Oxidative phase**

Step 1 : Glucose-6-phosphate is oxidized by $NADP^+$-dependent glucose-6-phosphate dehydrogenase (GPD). This produces 6-phosphogluconolactone and a molecule of NADPH (Nelson and Cox, 2005 ; Vasudevan et al., 2011; Stincone et al., 2014).

Step 2 : The lactone is hydrolyzed by gluconolactone hydrolase to form 6-phosphogluconic acid (Vasudevan et al., 2011).

Step 3 : This step involves oxidation coupled with decarboxylation and is catalyzed by 6-phosphogluconate dehydrogenase. In this reaction, 6-phosphogluconate is oxidized to form 3-keto-6-phosphogluconate, a transient intermediate that spontaneously undergoes decarboxylation to form ribulose-5-phosphate. The CO_2 carbon is derived from the COOH group of gluconic acid. During this step, a second molecule of NADPH is generated (Nelson and Cox, 2005; Vasudevan et al., 2011).

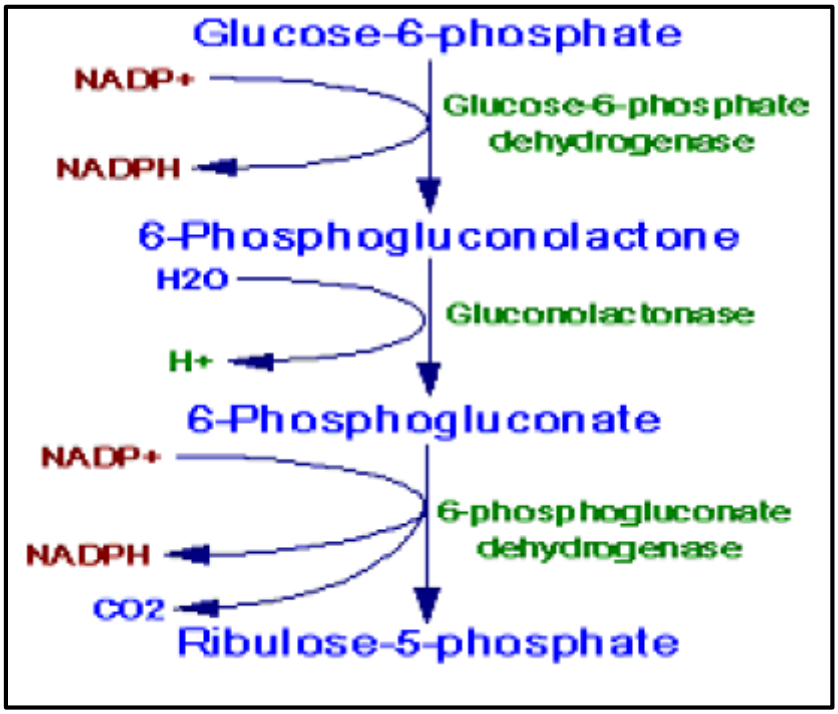

Figure 21: Oxidative phase of the pentose phosphate pathway (Solomon et al., 2004).

- **Non-oxidative phase**

Step 4 : Ribulose-5-phosphate is then isomerized to ribose-5-phosphate or epimerized to xylulose-5-phosphate (Nelson and Cox, 2005; Vasudevan et al., 2011; Stincone et al., 2014).

Step 5 : Transketolase, a thiamine pyrophosphate (TPP)-dependent enzyme, transfers a two-carbon unit (containing a keto group) from xylulose-5-phosphate to ribose-5-phosphate, producing sedoheptulose-7-phosphate (a seven-carbon sugar) and glyceraldehyde-3-phosphate (Nelson and Cox, 2005; Vasudevan et al., 2011; Stincone et al., 2014).

Step 6: This transaldolase-catalyzed group transfer reaction involves the transfer of a three-carbon unit from sedoheptulose-7-phosphate to glyceraldehyde-3-phosphate, forming fructose-6-phosphate and erythrose-4-phosphate (Nelson and Cox, 2005; Vasudevan et al., 2011; Stincone et al., 2014).

Step 7 : In another transketolase reaction, a 2C unit is transferred from xylulose-5-phosphate to erythrose-4-phosphate to form fructose-6-phosphate and glyceraldehyde-3-phosphate (Nelson and Cox, 2005; Vasudevan et al., 2011).

Step 8 : Two glyceraldehyde-3-phosphate molecules formed in step 7 are condensed to form fructose-6-phosphate (reversal of step 4 of glycolysis). The fructose-6-phosphate is then converted to glucose-6-phosphate (reversal of step 2 of glycolysis) (Vasudevan et al., 2011).

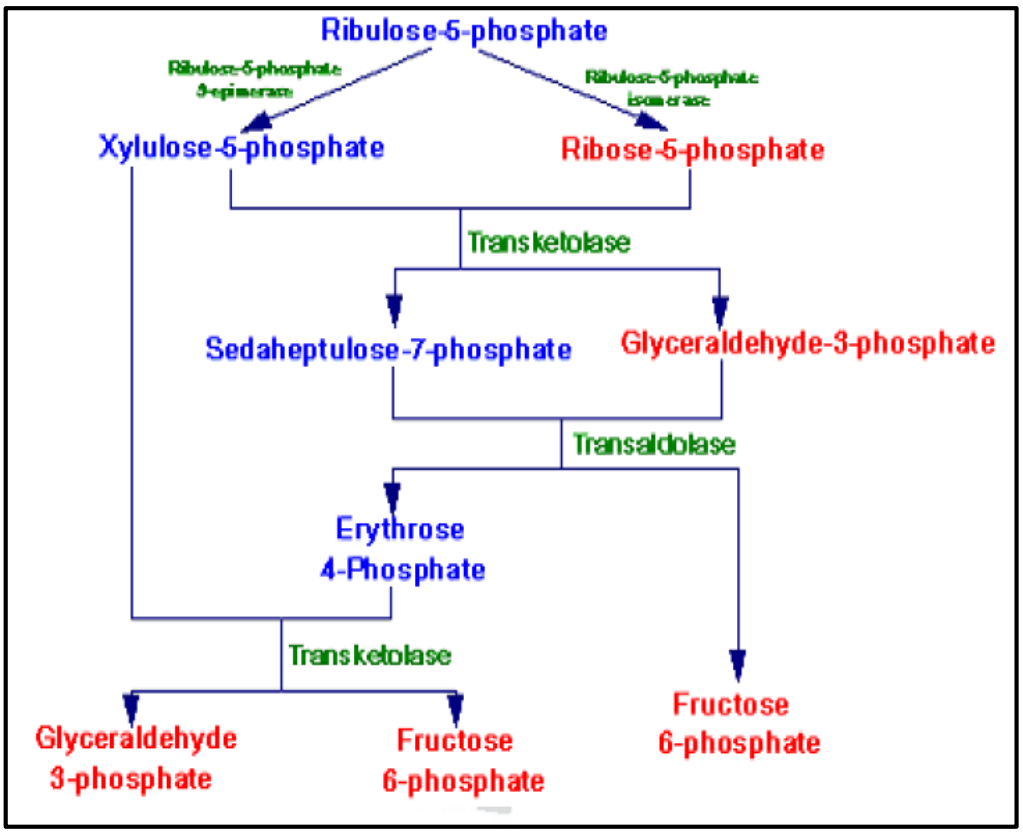

Figure 22: Non-oxidative phase of the pentose phosphate pathway (Solomon et al., 2004).

2.2. Lipid metabolism

- **Lipids**

Biological lipids are a group of chemically diverse compounds whose common and defining characteristic is their insolubility in water. The biological functions of lipids are as diverse as their chemical composition. Fats and oils are the primary forms of energy storage in many organisms. Phospholipids and sterols are major structural components of biological membranes. Other lipids, although present in relatively small quantities, play crucial roles as enzymatic cofactors, electron carriers, light-absorbing pigments, hydrophobic anchors for proteins, chaperones to help membrane proteins fold, emulsifying agents in the digestive tract, hormones, and intracellular messengers (Nelson and Cox, 2005).

During food ingestion, excess dietary non-esterified fatty acids are esterified into chemically inert triglycerides (TGs), which are then stored in cytosolic lipid droplets of adipocytes. When energy demand increases, triglyceride stores are mobilized by hydrolytic cleavage, and the resulting non-esterified fatty acids are delivered via the circulation to peripheral tissues for β-oxidation and ATP production. Essentially, every cell type can store TGs to some extent in intracellular organelles called lipid droplets (LDs) (Lass et al., 2011; Grabner et al., 2021).

2.2.1. Lipid catabolism

2.2.1.1. Lipolysis

a. Definition

Lipolysis is the biochemical pathway responsible for the catabolism of triglycerides stored in cellular lipid droplets into fatty acids and glycerol. This pathway is catalyzed by lipases. Fatty acids released from adipose tissue can enter the bloodstream and be absorbed by other organs as an energy source for β-oxidation and the subsequent generation of ATP. Furthermore, the released fatty acids and glycerol can also serve as substrates in the liver for ketogenesis and gluconeogenesis, respectively. Because of its central importance in lipid and energy homeostasis, lipolysis occurs in virtually all tissues and cell types, but it is more abundant in white and brown adipose tissues (Ahmadian et al., 2010; Lass et al., 2011; Grabner et al., 2021).

b. Steps of lipolysis

The hydrolysis of triglycerides into fatty acids and glycerol requires three consecutive steps involving at least three different enzymes:

• Triglyceride lipase catalyzes the initial stage of lipolysis, converting triglycerides into diacylglycerols;

• Hormone-sensitive lipase is primarily responsible for the hydrolysis of diacylglycerols into monoacylglycerols; and

• Monoacylglycerol lipase hydrolyzes monoacylglycerols into fatty acids and glycerol (Zechner et al., 2012).

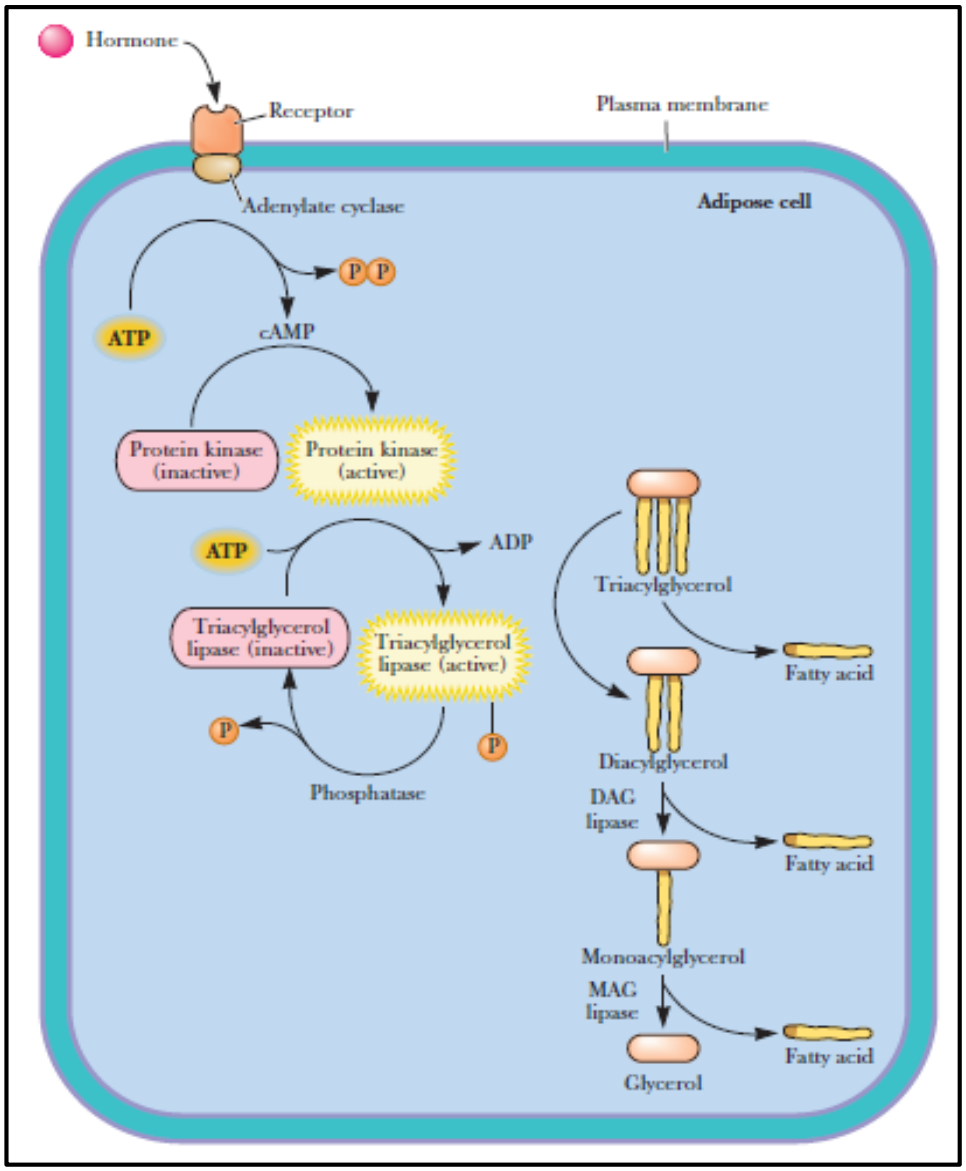

Figure 23: Release of fatty acids from triglycerides in adipose tissue (Campbell and Farrell, 2009).

2.2.1.2. β-oxidation

a. Definition

β-Oxidation can be defined as the oxidation of fatty acids at the β-carbon atom (Satyanarayana and Chakrapani, 2013). Fatty acid oxidation occurs in the mitochondria. Each step of fatty acid oxidation involves acyl-CoA derivatives and is catalyzed by distinct enzymes, uses NAD^+ and FAD as coenzymes, and generates ATP. This is an aerobic process, requiring the presence of oxygen (Murray et al., 2009). Fatty acids are oxidized by most tissues in the body. However, the brain, erythrocytes, and adrenal medulla cannot use fatty acids for their energy needs (Satyanarayana and Chakrapani, 2013).

b. Steps of β-oxydation

Fatty acid activation

Fatty acids are activated to acyl-CoA by thiokinases or acyl-CoA synthetases. The reaction occurs in two steps and requires ATP, coenzyme A, and Mg^{2+}. The fatty acid reacts with ATP to form acyladenylate, which then combines with coenzyme A to produce acyl-CoA. During activation, two high-energy phosphates are used, as ATP is converted to pyrophosphate (PPi). The inorganic enzyme pyrophosphatase hydrolyzes PPi to phosphate (Pi). The immediate removal of PPi makes this reaction completely irreversible (Campbell and Farrell, 2009; Murray et al., 2009; Satyanarayana and Chakrapani, 2013).

Transport of acyl-CoA to the mitochondria

The inner mitochondrial membrane is impermeable to fatty acids. A specialized carnitine transport system (carnitine shuttle) transports activated fatty acids from the cytosol to the mitochondria. This occurs in four steps :

1. The acyl group of acyl-CoA is transferred to carnitine, catalyzed by carnitine acyltransferase I (CAT-I) (present on the outer surface of the inner mitochondrial membrane).

2. Acyl-carnitine is transported across the membrane to the mitochondrial matrix by a specific carrier protein called translocase.

3. Carnitine acyltransferase II (CAT-II) (present on the inner surface of the inner mitochondrial membrane) converts acyl-carnitine to acyl-CoA.

4. The released carnitine returns to the cytosol for reuse (Vasudevan et al., 2011; Satyanarayana and Chakrapani, 2013).

- **β-oxidation of saturated fatty acids**

Each β-oxidation cycle, releasing a two-carbon unit, acetyl-CoA, occurs in a sequence of four reactions:

1. Oxidation : Acyl-CoA undergoes dehydrogenation by a flavoenzyme dependent on FAD, acyl-CoA dehydrogenase. A double bond forms between the α and β carbons (i.e., carbons 2 and 3).

2. Hydration : Enoyl-CoA hydratase hydrates the double bond to form hydroxyacyl-CoA.

3. Oxidation : Hydroxyaacyl-CoA dehydrogenase catalyzes the second oxidation and generates NADH. The product formed is β-ketoacyl-CoA.

4. Cleavage: The final β-oxidation reaction is the release of a 2-carbon molecule, acetyl CoA, and acyl CoA. This occurs via thiolytic cleavage catalyzed by β-ketoacyl CoA thiolase (or simply thiolase). The new acyl CoA, containing two fewer carbons than the original, re-enters the β-oxidation cycle. The process continues until the fatty acid is completely oxidized (Vasudevan et al., 2011; Satyanarayana and Chakrapani, 2013).

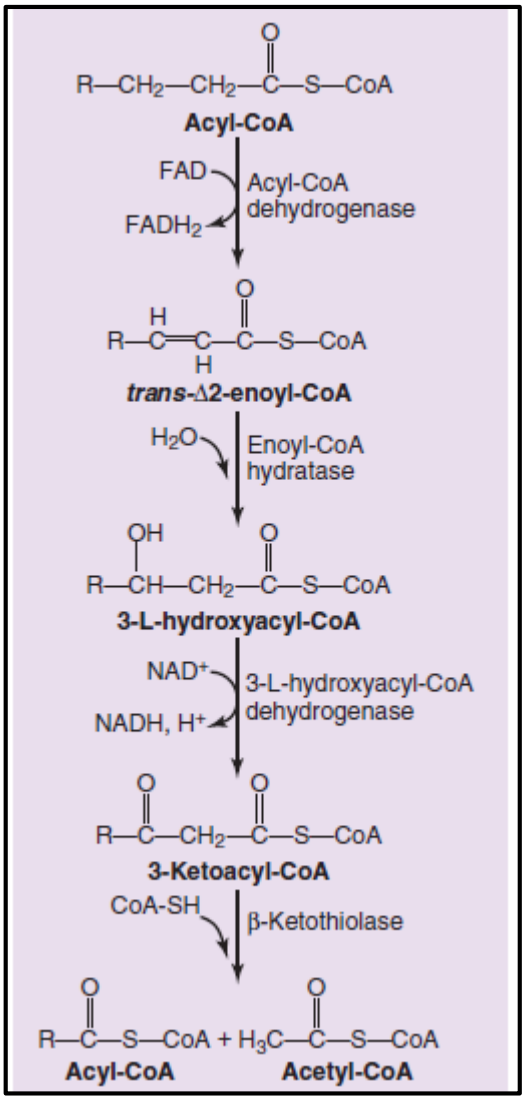

Figure 24: β-oxidation reactions (Meisenberg and Simmons, 2017).

c. Energy balance

Overall reaction for each oxidation-reduction cycle :

(Acyl CoA) 2n + (n-1) FAD + (n-1) NAD^+ + (n-1) H_2O + (n-1) CoASH → n Acétyl CoA + (n-1) $FADH_2$ + (n-1) NADH + (n-1) H^+.

***Example: Oxidation of palmitoyl CoA**

The summary of β-oxidation of palmitoyl CoA is presented below:

Palmitoyl CoA + 7 CoASH + 7 FAD + 7 NAD^+ + 7H_2O→ 8 Acétyl CoA + 7 $FADH_2$ + 7 NADH + 7H^+.

Palmitoyl CoA undergoes seven β-oxidation cycles to produce eight acetyl CoA molecules. Acetyl CoA can enter the citric acid cycle and be completely oxidized to CO_2 and H_2O. The energy obtained from the complete oxidation of palmitic acid (16 carbons) is given in Table 3 and figure 25 (Satyanarayana and Chakrapani, 2013).

Table 3: Energy balance of palmitic acid oxidation (Satyanarayana and Chakrapani, 2013).

Mechanism	**ATP Yield**
I. 7 cycles of β-oxidation	
7 FADH2	14
7 NADH	21
II. From 8 acetyl CoA	
Oxidized by the citric acid cycle, each acetyl CoA yields 12 ATP	96
Total energy of one molecule of palmitoyl CoA	131
Energy used for activation (formation of palmitoyl CoA)	–2
Net yield for one molecule of palmitate	129

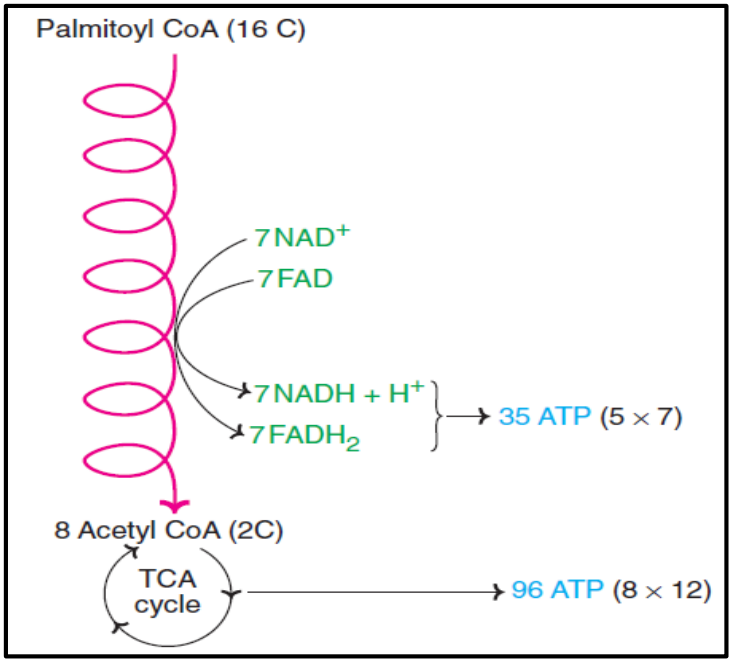

Figure 25: Overview of palmitic acid oxidation (Satyanarayana and Chakrapani, 2013).

d. β-oxidation of odd-numbered saturated fatty acids

The β-oxidation of saturated fatty acids containing an odd number of carbon atoms proceeds in the same way as that of fatty acids with an even number of carbons. The only difference is that, during the last β-oxidation cycle, a three-carbon fragment is left (instead of a two-carbon unit for saturated fatty acids). This compound is propionyl-CoA, which is converted to succinyl-CoA as follows:

1. Propionyl CoA is carboxylated in the presence of ATP, CO_2, and vitamin biotin to D-methylmalonyl CoA.

2. Methylmalonyl-CoA racemase converts methylmalonyl-CoA to its L-form. This reaction (D → L) is essential for this compound to enter the body's metabolic processes.

3. The next enzyme, methylmalonyl-CoA mutase, is dependent on vitamin B12 (deoxyadenosylcobalamin). It catalyzes the conversion of methylmalonyl-CoA (a branched compound) to succinyl-CoA (a linear-chain compound), which can then enter the citric acid cycle (Satyanarayana and Chakrapani, 2013).

e. β-oxidation of unsaturated fatty acids

Due to the presence of double bonds, unsaturated fatty acids are not reduced to the same extent as saturated fatty acids. Consequently, the oxidation of unsaturated fatty acids generally provides less energy than that of saturated fatty acids.

Most of the reactions involved in the oxidation of unsaturated fatty acids are identical to those observed during the β-oxidation of saturated fatty acids. However, the presence of double bonds presents an obstacle to β-oxidation. This problem is solved by two additional enzymes : an isomerase and an epimerase (Satyanarayana and Chakrapani, 2013).

2.2.2. Fatty acid biosynthesis (lipogenesis)

A. Definition

Dietary carbohydrates and amino acids, when consumed in excess, can be converted into fatty acids and stored as triacylglycerols (triglycerides). Lipogenesis is the synthesis of fatty acids from a two-carbon precursor, acetyl-CoA. It occurs primarily in adipose tissue, but also in the liver, kidneys, muscles, heart, lactating mammary gland, and pancreas (Satyanarayana and Chakrapani, 2013; Saponaro et al., 2015; Woo et al., 2016).

B. Steps of lipogenesis

- **Production of acetyl CoA and NADPH**

Acetyl CoA and NADPH are prerequisites for fatty acid synthesis. Acetyl CoA is produced in the mitochondria by the oxidation of pyruvate and fatty acids, the breakdown of the carbon skeleton of certain amino acids, and from ketone bodies. The mitochondria are not permeable to acetyl CoA. An alternative or bypass pathway is in place for the transfer of acetyl CoA to the cytosol. Acetyl CoA condenses with oxaloacetate in the mitochondria to form citrate. Citrate is transported to the cytosol where it is cleaved by citrate lyase to release acetyl CoA and oxaloacetate. Oxaloacetate in the cytosol is converted to malate. The malic enzyme converts malate to pyruvate. This reaction generates NADPH and CO_2. These two compounds are used for fatty acid synthesis (Satyanarayana and Chakrapani, 2013).

- **Formation of malonyl CoA**

In the cytosol, acetyl-CoA is carboxylated, producing malonyl-CoA, a key intermediate in fatty acid biosynthesis. This reaction is catalyzed by acetyl-CoA carboxylase (Campbell and Farrell, 2009; Vasudevan et al., 2011).

- **Fatty acid synthase complex reactions**

The remaining reactions of fatty acid synthesis are catalyzed by a multifunctional enzyme known as fatty acid synthase complex. In eukaryotic cells, including humans, fatty acid synthase complex exists as a dimer consisting of two identical units. Each monomer possesses the activities of seven different enzymes and an acyl carrier protein (ACP) linked to 4′-phosphopantetheine (Satyanarayana and Chakrapani, 2013).

a. Transfer of acetyl and malonyl groups

Acetyl transacylase catalyzes the transfer of the acetyl group to the cysteine (SH) group of the other monomer in the fatty acid synthase complex. One molecule of acetyl-CoA and one molecule of malonyl-CoA bind to the multi-enzyme complex. Malonyl transacylase transfers the malonyl group to the SH group of the PCA of one monomer of the enzyme (Vasudevan et al., 2011).

b. Condensation

The acetyl (2C) and malonyl (3C) units are condensed to form beta-ketoacyl-ACP or acetoacetyl-ACP (4C). During this process, one carbon atom is lost as CO_2. The enzyme is called ketoacyl synthase (Vasudevan et al., 2011).

c. Reduction

Acetoacetyl-ACP is reduced by NADPH-dependent beta-ketoacyl reductase to form β-hydroxybutyryl-ACP (Vasudevan et al., 2011).

d. Dehydration

β-hydroxybutyryl-ACP is then dehydrated by a dehydratase (β-hydroxyacyl-ACP dehydratase) to form enoyl-ACP (Vasudevan et al., 2011).

e. Second reduction

Enoyl-ACP is further reduced by enoyl reductase using a second molecule of NADPH to form butyryl-ACP. The butyryl group (4C) is then transferred to the SH group of the condensation enzyme on the other monomer, and a second molecule of malonyl CoA binds to the phosphopantothenyl SH group. The reaction sequence : condensation, reduction, dehydration, and reduction (steps b, c, d, e) is repeated. The cycles are repeated a total of seven times until the 16-carbon palmitic acid is formed (Vasudevan et al., 2011).

f. Release of palmitic acid (palmitate)

Thioesterase or deacylase (TE) activity releases palmitate from the multienzyme complex. The end product is palmitic acid (16C) in the liver and adipose tissue. However, in the lactating mammary gland, the end products are capric acid (10C) and lauric acid (12C). Breast milk contains these medium-chain fatty acids (Vasudevan et al., 2011).

2.3. Ketone bodies metabolism

2.3.1. Definition of ketone bodies

Substances derived from acetone, known as "ketone bodies," are produced when an excess of acetyl-CoA results from β-oxidation. This occurs when there is insufficient oxaloacetate available to react with the large amounts of acetyl-CoA that could enter the citric acid cycle. Oxaloacetate, originates from glycolysis, as it is formed from pyruvate in a reaction catalyzed by pyruvate carboxylase. Such a situation can occur when an organism has a high lipid and low carbohydrate intake, but can also occur in starvation **or** diabetes (Campbell and Farrell, 2009). Ketone bodies are a group of water-soluble molecules containing ketone groups, consisting of acetoacetate (AcAc), β-hydroxybutyrate (βHB), and acetone. Among ketone bodies, β-hydroxybutyrate is the most abundant, representing approximately 70% of the circulating ketone body pool. βHB and AcAc are important alternative energy sources for extrahepatic tissues (Hwang et al., 2022).

2.3.2. Ketone body biosynthesis (ketogenesis)

2.3.2.1. Definition

Ketogenesis is a metabolic pathway that produces ketone bodies, which provide an alternative form of energy to the body (Dhillon et al., 2023). Ketone bodies are produced in the mitochondria of hepatocytes from acetyl-CoA derived from the β-oxidation of fatty acids, and they are transported to extrahepatic tissues for final oxidation. This physiological process provides an alternative fuel source that is enhanced by relatively short periods of fasting, which increase fatty acid availability and decrease carbohydrate availability (Puchalska and Crawford, 2017; Matsuura et al., 2023).

2.3.2.2. Steps of ketogenesis

Ketogenesis occurs through a series of enzymatic reactions:

• Two molecules of acetyl-CoA are condensed into acetoacetyl-CoA by the enzyme acetoacetyl-CoA thiolase (acetyl-CoA acetyltransferase 1).

• Acetoacetyl-CoA is converted into hydroxymethylglutaryl-CoA by the enzyme 3-hydroxy-3-methylglutaryl-CoA synthase 2 (HMGCS2).

• Hydroxymethylglutaryl-CoA lyase (HMGCL) then cleaves hydroxymethylglutaryl-CoA to release acetyl-CoA and acetoacetate.

• β-Hydroxybutyrate (βOHB) is generated from acetoacetate by the phosphatidylcholine-dependent enzyme β-hydroxybutyrate dehydrogenase 1 (BDH1) (Hwang et al., 2022; Mooli and Ramakrishnan, 2022).

• Acetoacetate can be converted to acetone by non-enzymatic decarboxylation (Dhillon et al., 2023).

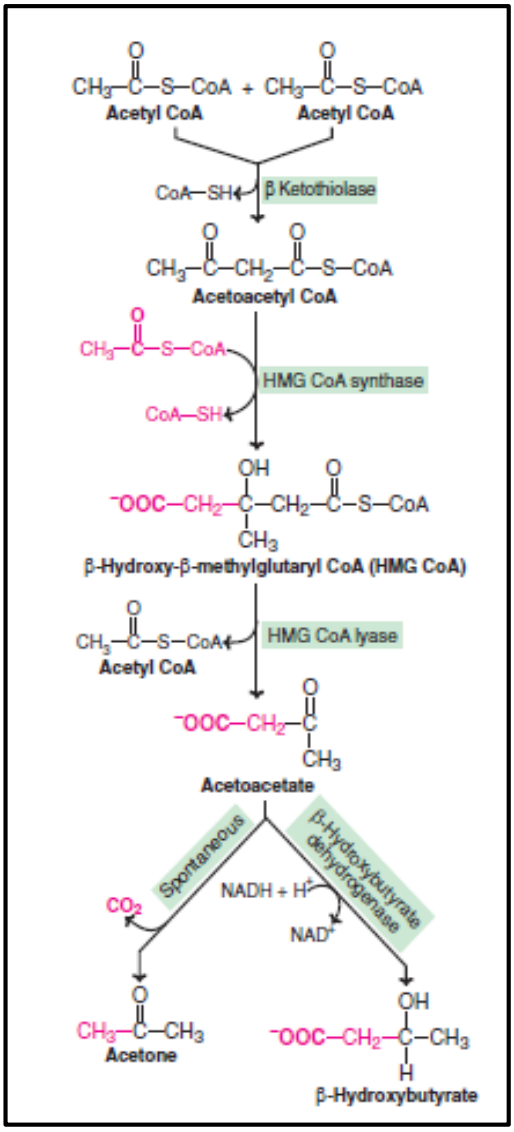

Figure 26: Ketogenesis (Satyanarayana and Chakrapani, 2013).

2.3.3. Ketone body degradation (ketolysis)

2.3.3.1. Definition

Ketolysis is the process by which ketone bodies are broken down to provide energy to tissues. It occurs in the mitochondria of many organs (except the liver, which cannot use ketone bodies) (Leonard, 2020).

2.3.3.2. Steps of ketolysis

-Oxidation of βOHB to acetoacetate by mitochondrial BDH1.

-Acetatoacetate is converted to acetoacetate-CoA by succinyl-CoA:3-oxoacid-CoA transferase (SCOT).

-Acetatoacetate-CoA is cleaved by mitochondrial acetoacetyl-CoA thiolase, producing two molecules of acetyl-CoA (Puchalska and Crawford, 2017; Kolb et al., 2021; Hwang et al., 2022).

Acetyl-CoA enters the Krebs cycle, lipogenesis or is excreted in the urine (Mooli and Ramakrishnan, 2022).

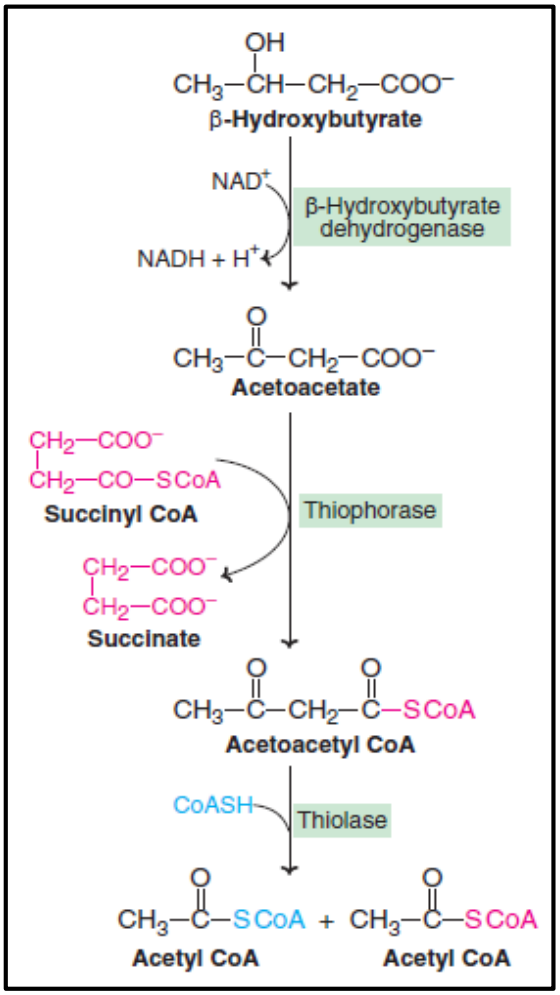

Figure 27: Ketolysis (Satyanarayana and Chakrapani, 2013).

2.4. Amino acid metabolism

2.4.1. Proteins

Proteins are the most abundant biological macromolecules, present in all cells and all parts of cells. Proteins are also very diverse; thousands of different types, ranging from relatively small peptides to enormous polymers with molecular weights in the millions, can be found in a single cell. Proteins are polymers of amino acids, with each amino acid residue linked to its neighbor by a specific type of covalent bond (Nelson and Cox, 2005).

Protein digestion begins in the stomach, where the acidic environment promotes protein denaturation. The main proteolytic enzyme in the stomach is pepsin (a non-specific protease). Protein breakdown continues in the intestinal lumen due to the activity of proteolytic enzymes secreted by the pancreas. These proteins are secreted as inactive zymogens and then converted into active enzymes. Substrates are broken down into free amino acids as well as di- and tripeptides. Digestion is further enhanced by proteases, such as aminopeptidase N, which are

located in the plasma membrane of intestinal cells. Simple amino acids, along with di- and tripeptides, are transported from the lumen into intestinal cells and then released into the bloodstream for absorption by other tissues (Berg et al., 2002).

Twenty different amino acids are commonly found in proteins. The 20 most common amino acids are α-amino acids. They have a carboxyl group and an amine group bonded to the same carbon atom (α-carbon). They differ from one another by their side chains, or R groups, which vary in structure, size, and electrical charge, and which influence the solubility of the amino acids in water (Nelson and Cox, 2005). Animals cannot synthesize nine amino acids; these are called "essential" amino acids and must therefore be obtained from food. The other 11 amino acids are "non-essential" or "conditionally essential" (Chandel, 2021).

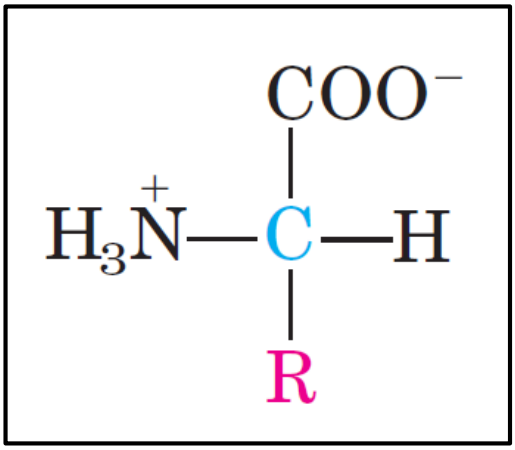

Figure 28: General structure of an amino acid (Nelson and Cox, 2005).

2.4.2. Amino acid catabolism

There is no storage form for amino acids as there is for carbohydrates (glycogen) and lipids (triglycerides). Excess absorbed amino acids are metabolized (Vasudevan et al., 2011; Satyanarayana and Chakrapani, 2013). That is, free amino acids produced from the breakdown of cellular or dietary proteins are deaminated to produce NH_4^+ and a carbon skeleton. The NH_4^+ enters the urea cycle, and the carbon skeleton can enter metabolic pathways to generate ATP, glucose, and fatty acids (Chandel, 2021).

2.4.2.1. Transamination

The transfer of an amino group (NH_2) from an amino acid to a ketone acid is called transamination. This process involves the interconversion of an amino acid and a ketone acid, catalyzed by a group of enzymes called transaminases (aminotransferases). These enzymes require pyridoxal phosphate, a coenzyme derived from vitamin B6. Specific transaminases exist for different amino acid–keto acid pairs, but only aspartate transaminase and alanine

transaminase play major roles in transamination. The transamination reaction is reversible (Vasudevan et al., 2011; Satyanarayana and Chakrapani, 2013).

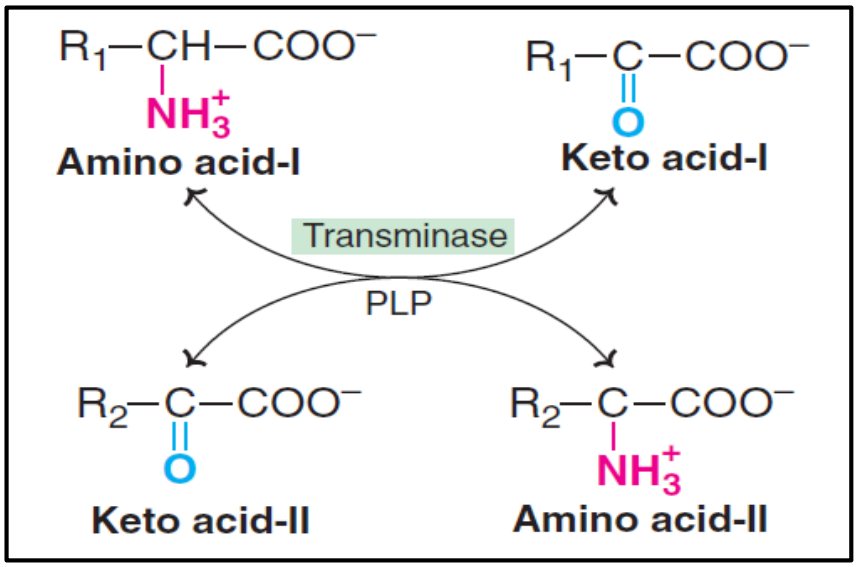

Figure 29: Transamination reaction (Satyanarayana and Chakrapani, 2013).

-Transamination is crucial for the redistribution of amino groups and the production of non-essential amino acids, according to the cell's needs. It involves both the catabolism (breakdown) and anabolism (synthesis) of amino acids.

-Transamination diverts excess amino acids toward energy production.

-Amino acids undergo transamination, ultimately concentrating nitrogen in glutamate. Glutamate is the only amino acid that undergoes significant oxidative deamination to release free NH_3 for urea synthesis (Satyanarayana and Chakrapani, 2013).

2.4.2.2. Deamination

-Deamination involves the removal of the amine group from amino acids in the form of NH_3. Deamination releases ammonia for urea synthesis. Simultaneously, the carbon skeleton of amino acids is converted into ketone bodies. Deamination can be oxidative or non-oxidative.

-Transamination and deamination often involve glutamate as the central molecule (Satyanarayana and Chakrapani, 2013).

- **Oxidative deamination**

Oxidative deamination is the release of free ammonia from the amine group of amino acids associated with oxidation. This occurs primarily in the liver and kidneys. The purpose of oxidative deamination is to provide NH3 for the synthesis of urea and α-keto acids for various reactions, including energy production (Satyanarayana and Chakrapani, 2013).

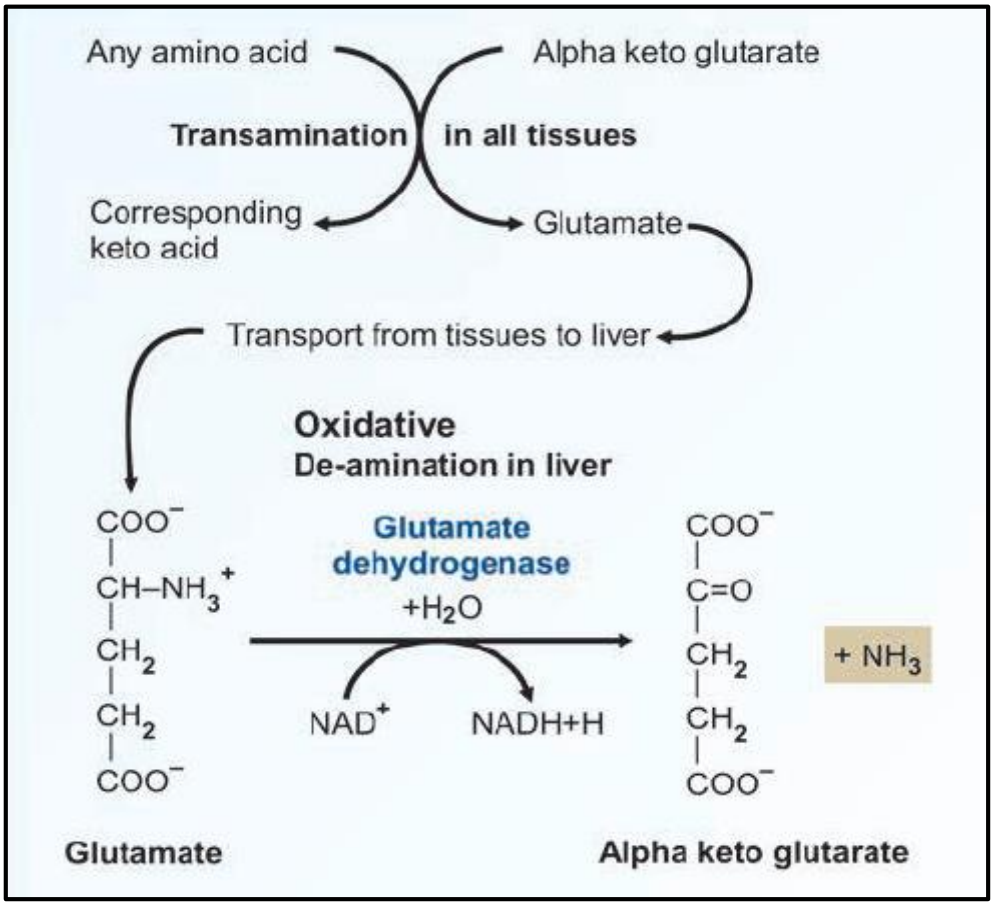

Figure 30: Trans-deamination (Vasudevan et al., 2011).

➢ **Glutamate dehydrogenase :** During the transamination process, amino groups from most amino acids are transferred to α-ketoglutarate to produce glutamate. Glutamate rapidly undergoes oxidative deamination, catalyzed by glutamate dehydrogenase (GDH), to release ammonia. This enzyme can use either NAD^+ or $NADP^+$ as a coenzyme (Satyanarayana and Chakrapani, 2013).

-After ingesting a protein-rich meal, the level of glutamate in the liver is elevated. It is converted into α-ketoglutarate with the release of NH_3. Furthermore, when cellular energy levels are low, the breakdown of glutamate is increased to provide α-ketoglutarate, which enters the TCA cycle to release energy (Satyanarayana and Chakrapani, 2013).

- **Non-oxidative deamination**

Some amino acids can be deaminated to release NH_3 without undergoing oxidation.

- Amino acid dehydrases : Serine and threonine are hydroxylated amino acids. They undergo non-oxidative deamination catalyzed by PLP-dependent dehydrases (dehydratases) : serine dehydratase and threonine dehydratase (serine is converted to pyruvate and threonine to α-

ketobutyric acid) (Berg et al., 2002; Vasudevan et al., 2011; Satyanarayana and Chakrapani, 2013).

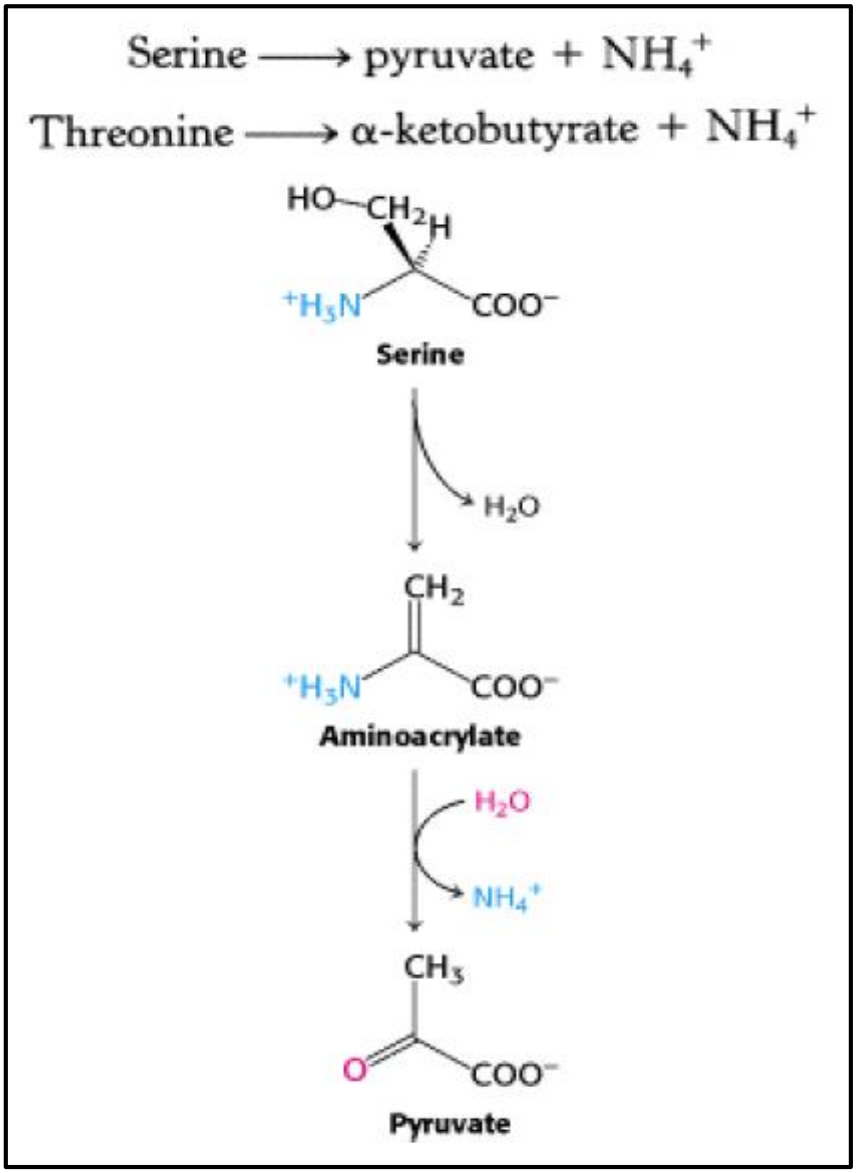

Figure 31: Deamination of serine and threonine (Berg et al., 2002).

-Desulfhydrase : cysteine undergoes simultaneous deamination and transsulfuration to form pyruvate (Vasudevan et al., 2011).

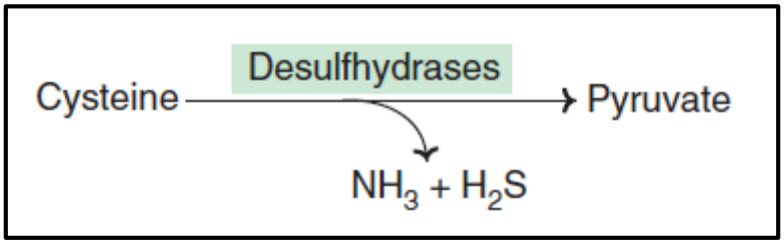

Figure 32: Transsulfuration of cysteine (Satyanarayana and Chakrapani, 2013).

-Histidine deamination : Histidine also undergoes non-oxidative deamination to form urocanic acid; catalyzed by histidase (Vasudevan et al., 2011).

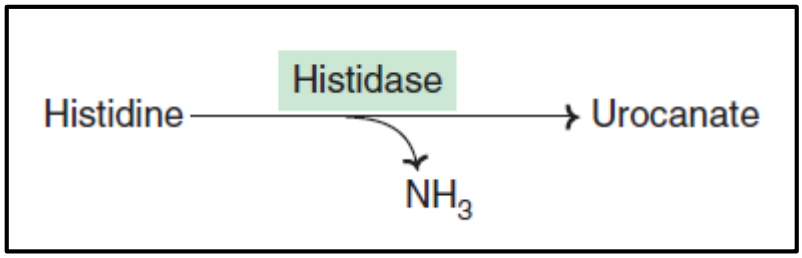

Figure 33: Deamination of histidine (Satyanarayana and Chakrapani, 2013).

2.4.2.3. NH_3 Transport

-Ammonia is transported between different tissues and the liver primarily in the form of glutamine or alanine, not as free ammonia. Alanine is important for the transport of NH_3 from muscle to the liver. Glutamine serves as a storage and transport form of NH_3. Its synthesis occurs mainly in the liver, brain, and muscles. Ammonia is eliminated from the brain primarily in the form of glutamine. Glutamine is freely diffusible into tissues and therefore easily transported.

- Glutamine synthetase (a mitochondrial enzyme) is responsible for the synthesis of glutamine from glutamate and ammonia. This reaction is unidirectional and requires ATP and Mg^{2+} ions.

-Glutamine can be deaminated by hydrolysis to release ammonia by glutaminase, an enzyme mainly present in kidney and intestinal cells (Satyanarayana and Chakrapani, 2013).

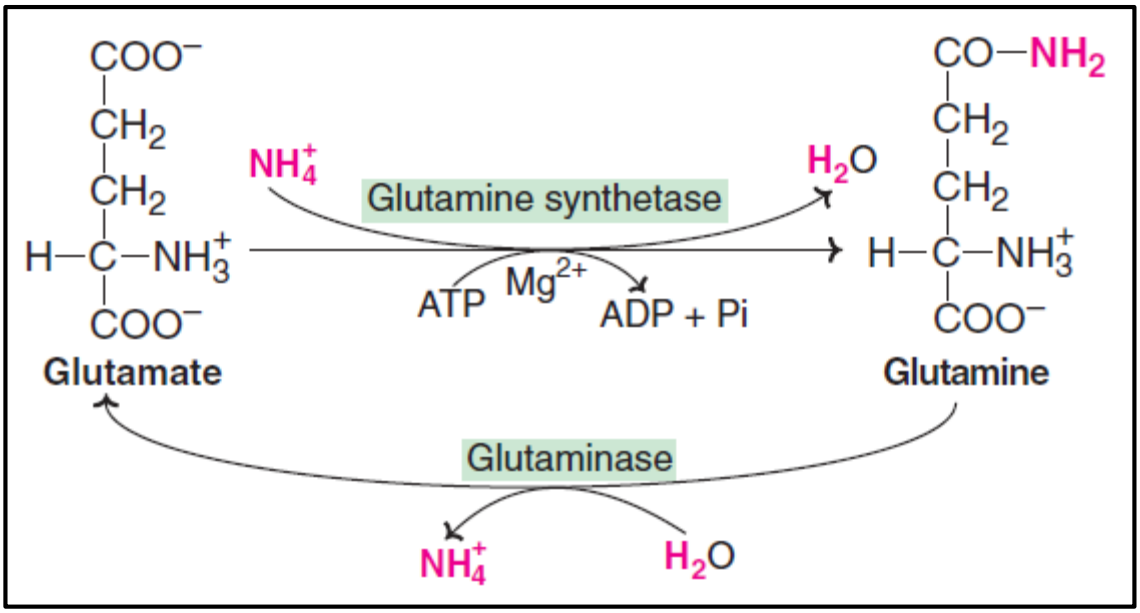

Figure 34: Synthesis of glutamine and its conversion to glutamate (note: the reactions are independent and irreversible) (Satyanarayana and Chakrapani, 2013).

2.4.2.4. Urea cycle

Urea is the end product of protein metabolism (amino acid metabolism). Nitrogen from amino acids, transformed into ammonia, is toxic to the body. It is converted into urea and detoxified.

Urea is synthesized in the liver and transported to the kidneys to be excreted in the urine (Satyanarayana and Chakrapani, 2013).

A. Mitochondrial phase

- **Carbamoyl phosphate formation**

Mitochondrial carbamoyl phosphate synthase I (CPS I) catalyzes the condensation of NH_4^+ ions with CO_2 to form carbamoyl phosphate. This step consumes two ATP molecules and is irreversible and rate-limiting. CPS I requires N-acetylglutamate for its activity. Another enzyme, carbamoyl phosphate synthase II (CPS II), involved in pyrimidine synthesis, is present in the cytosol. It accepts the amino group from glutamine and does not require N-acetylglutamate for its activity (Murray et al., 2009; Vasudevan et al., 2011; Satyanarayana and Chakrapani, 2013; Meisenberg and Simmons, 2017).

- **Citrulline formation**

Citrulline is synthesized from carbamoyl phosphate and ornithine by ornithine transcarbamoylase. Ornithine is regenerated and used in the urea cycle. Both ornithine and citrulline are basic amino acids. (They are never found in protein structures due to the absence of codons.) The citrulline produced in this reaction is transported to the cytosol (Murray et al., 2009; Vasudevan et al., 2011; Satyanarayana and Chakrapani, 2013).

B. Cytosolic phase

- **Arginosuccinate formation**

Arginosuccinate synthase condenses citrulline with aspartate to produce arginosuccinate. The second amine group of urea is incorporated in this reaction. This step requires ATP, which is cleaved into AMP and pyrophosphate (PPi). The latter is immediately decomposed into inorganic phosphate (Pi) (Berg et al., 2002; Murray et al., 2009; Vasudevan et al., 2011; Satyanarayana and Chakrapani, 2013).

- **Cleavage of arginosuccinate**

Arginosuccinase cleaves argininesuccinate to give arginine and fumarate. Arginine is the immediate precursor of urea. The fumarate released here provides a link with the TCA cycle, gluconeogenesis, etc. (Berg et al., 2002; Murray et al., 2009; Vasudevan et al., 2011; Satyanarayana and Chakrapani, 2013).

- **Urea formation**

Arginase is the fifth and final enzyme that cleaves arginine to produce urea and ornithine. The regenerated ornithine enters the mitochondria to be reused in the urea cycle. Arginase is

activated by CO_2^+ and Mn^{2+}. Ornithine and lysine compete with arginine (competitive inhibition). Arginase is found primarily in the liver, while the remaining four enzymes of the urea cycle are also present in other tissues. For this reason, arginine synthesis can occur to varying degrees in many tissues. However, only the liver can ultimately produce urea (Berg et al., 2002; Murray et al., 2009; Vasudevan et al., 2011; Satyanarayana and Chakrapani, 2013).

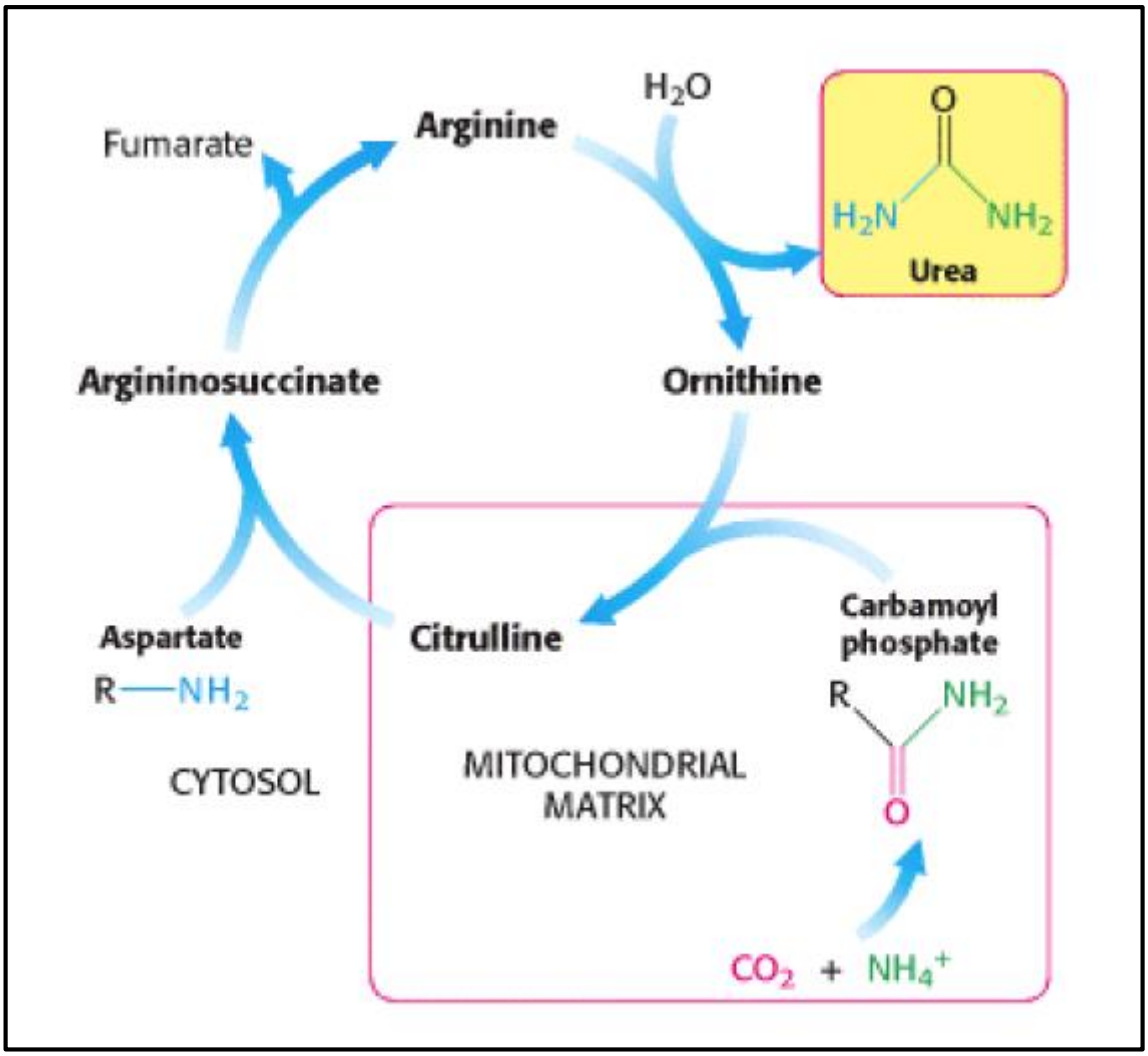

Figure 35: Urea cycle (Berg et al., 2002).

2.4.2.5. Fate of the carbon skeleton of amino acids

The breakdown of amino acids involves transforming their carbon skeleton into major metabolic intermediates that can be converted into glucose or oxidized by the citric acid cycle. The carbon skeleton of the diverse set of 20 amino acids lead to the formation of seven molecules : pyruvate, acetyl CoA, acetoacetyl CoA, α-ketoglutarate, succinyl CoA, fumarate, and oxaloacetate. Amino acids broken down into acetyl-CoA or acetoacetyl-CoA are called ketogenic amino acids because they can give ketone bodies or fatty acids. Amino acids degraded into pyruvate, α-ketoglutarate, succinyl-CoA, fumarate or oxaloacetate are called

glucogenic amino acids because they can be converted into glucose (Berg et al., 2002; Campbell and Farrell, 2009).

- Ketogenic amino acids : Leucine and lysine
- Mixed amino acids (ketogenic and glucogenic) : Isoleucine, phenylalanine, tryptophan and tyrosine.
- Glucogenic amino acids : The other 14 amino acids (threonine, glycine, serine, cysteine, alanine, asparagine, aspartate, valine, methionine, histidine, arginine, proline, glutamine, glutamate) are classified as glucogenic (Berg et al., 2002).

2.4.3. Amino acid synthesis

Amino acid biosynthesis involves several biochemical pathways in which amino acids are assembled from other precursors generated in three different metabolic pathways : the glycolytic pathway, the pentose phosphate pathway, and the Krebs cycle (Deferrari et al., 2010). Amino acids are grouped into families based on common precursors :

- **α-ketoglutarate :** Gives the glutamate family (glutamate, glutamine, proline and arginine).
- **Oxaloacetate :** Gives the aspartate family (aspartate, asparagine, methionine, threonine, isoleucine and lysine).
- **3-phosphoglycerate :** Gives the serine family (serine, cysteine, glycine).
- **Pyruvate :** Gives the alanine family (alanine, valine and leucine).
- **Phosphoenolpyruvate and erythrose-4-phosphate :** Give the family of aromatic amino acids (phenylalanine, tyrosine and tryptophan).
- **Ribose-5-phosphate :** Gives the histidine family (histidine) (Campbell and Farrell, 2009).

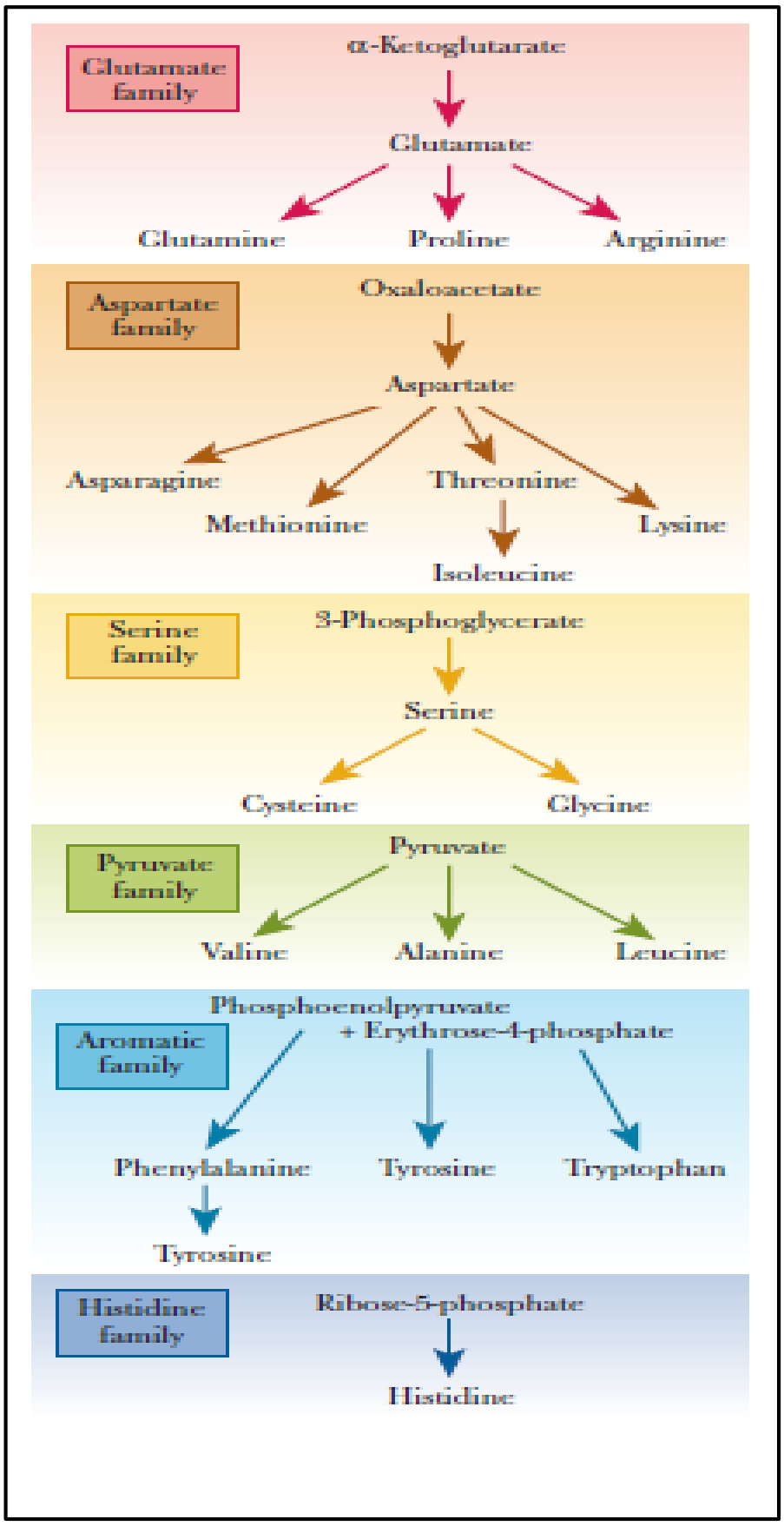

Figure 36: Amino acid families based on biosynthetic pathways (Campbell and Farrell, 2009).

CHAPTER 2 : REGULATION OF METABOLIC PATHWAYS AND THEIR DEVIATIONS - FUNCTIONAL EXPLORATION

1. Types of regulation

Metabolic pathways are regulated mainly through two key mechanisms : enzymatic regulation, which controls the activity of individual enzymes inside cells, and hormonal regulation, which coordinates metabolism across the entire body. Together, these mechanisms keep metabolism balanced and allow it to adapt to changing needs.

1.1. Enzyme regulation

-Some enzymes are covalently modified, usually by phosphorylation of amino acid side chains. The phosphorylated or dephosphorylated enzyme is the catalytically active form. This regulatory mechanism requires a protein kinase (phosphorylation enzyme) and a protein phosphatase (dephosphorylation enzyme).

-Some enzymes are regulated by allosteric effectors. In most cases, the allosteric effector is a substrate, an intermediate, or a product of the pathway (Meisenberg and Simmons, 2017).

1.2. Hormonal regulation

The second role of metabolic regulation is the maintenance of homeostasis within the organism (homeostasis is the ability or tendency to maintain the internal stability of an organism despite environmental changes).This stability is ensured through the action of specific hormones, which coordinate metabolic activities across different tissues and organs.

-Insulin is released after a carbohydrate-rich meal and promotes the uptake and use of these nutrients by the body.

-Glucagon is released during fasting and helps maintain an adequate blood glucose level.

- Epinephrine (adrenaline) and norepinephrine (noradrenaline) are released during acute stress and physical exertion. They stimulate the mobilization of stored fats and glycogen.

- Cortisol is released during more prolonged stress, when it promotes glucose synthesis and fat breakdown (Meisenberg and Simmons, 2017).

2. Regulation of metabolic pathways

2.1. Regulation of glycolysis

2.1.1. Allosteric regulation

The three enzymes, namely hexokinase (and glucokinase), phosphofructokinase and pyruvate kinase, catalyzing irreversible reactions, regulate glycolysis.

-Hexokinase is inhibited by glucose-6-phosphate. Glucokinase, which specifically phosphorylates glucose, is an inducible enzyme. The glucose substrate, probably through the involvement of insulin, induces glucokinase (Satyanarayana and Chakrapani, 2013).

- Phosphofructokinase (PFK) is the most important regulatory enzyme of glycolysis. This enzyme catalyzes the rate-limiting commitment step of glycolysis. PFK is an allosteric enzyme regulated by allosteric effectors. ATP, citrate, and H+ ions (low pH) are the most important allosteric inhibitors, while fructose-2,6-bisphosphate, ADP, AMP, and Pi are the allosteric activators (Satyanarayana and Chakrapani, 2013).

- Pyruvate kinase also regulates glycolysis. This enzyme is inhibited by ATP and activated by F-1,6-BP. Pyruvate kinase is active (a) in the dephosphorylated state and inactive (b) in the phosphorylated state. Inactivation of pyruvate kinase by phosphorylation is induced by cAMP-dependent protein kinase (Satyanarayana and Chakrapani, 2013).

2.1.2. Hormonal regulation

-Insulin promotes glycolysis by activating three key glycolytic enzymes (hexokinase, phosphofructokinase (PFK), and pyruvate kinase) (Vasudevan et al., 2011).

-Glucagon and glucocorticoids inhibit glycolysis and promote gluconeogenesis (Vasudevan et al., 2011).

2.2. Regulation of the Krebs cycle

2.2.1. Allosteric regulation

-Citrate and citrate synthase : The formation of citrate from acetyl CoA is an important part of the control. ATP acts as an allosteric inhibitor of citrate synthase. Citrate allosterically inhibits PFK, the key enzyme of glycolysis ; stimulates fructose-1,6-bisphosphatase, a key enzyme of gluconeogenesis ; and activates acetyl CoA carboxylase, a key enzyme in fatty acid synthesis.

-When NADH and $FADH_2$ accumulate, they inhibit the TCA cycle.

-α-ketoglutarate dehydrogenase : Is inhibited by succinyl CoA and NADH.

-Isocitrate dehydrogenase : ADP acts as a positive modifier, enhancing substrate binding and NADH acts as an inhibitor (Vasudevan et al., 2011).

2.3. Regulation of gluconeogenesis

2.3.1. Allosteric regulation

-Pyruvate carboxylase : Is an allosteric enzyme. Acetyl-CoA acts as an activator, promoting the production of oxaloacetate when acetyl-CoA levels are sufficiently high.

-Fructose-1,6-bisphosphatase : Citrate is an activator, while fructose-2,6-bisphosphate and AMP are inhibitors. These three effectors have exactly opposite effects on phosphofructokinase (PFK).

- ATP: Gluconeogenesis is stimulated by ATP.

2.3.2. Hormonal regulation

- Influence of glucagon : Glucagon stimulates gluconeogenesis through two mechanisms :

  - The active form of pyruvate kinase is converted to its inactive form via cyclic AMP, triggered by glucagon. As a result, the conversion of phosphoenolpyruvate (PEP) to pyruvate decreases, and PEP is redirected toward gluconeogenesis.

  - Glucagon reduces the concentration of fructose-2,6-bisphosphate. This compound allosterically inhibits phosphofructokinase and activates fructose-1,6-bisphosphatase, as a result, gluconeogenesis is promoted (Vasudevan et al., 2011; Satyanarayana and Chakrapani, 2013).

- Insulin : Inhibits gluconeogenesis. It can act by directly activating or deactivating enzymes that limit the availability of the gluconeogenic substrate or by acting on gluconeogenic activators (Sahoo et al., 2023).

- Cortisol : A major physiological role of cortisol is considered to be gluconeogenesis and the maintenance of blood glucose (Hucklebridge et al., 1999).

2.4. Regulation of glycogenesis and glycogenolysis

2.4.1. Allosteric regulation

Allosteric regulation plays a crucial role in glycogen metabolism by modulating the activities of glycogen synthase and glycogen phosphorylase according to energy and substrate availability. When energy levels are high and glucose-6-phosphate is abundant, glycogen synthase is activated to promote glycogen synthesis. Conversely, when glucose concentrations and energy levels are low, glycogen phosphorylase is activated to break down glycogen. More specifically, glucose-6-phosphate, ATP, and free glucose in the liver inhibit glycogen phosphorylase (Satyanarayana and Chakrapani, 2013).

2.4.2. Hormonal regulation

The hormonal regulation of glycogen metabolism involves covalent modifications, primarily the phosphorylation and dephosphorylation of enzymes that control glycogen synthesis and degradation. Hormones such as epinephrine, norepinephrine, and glucagon stimulate adenylate cyclase, thereby increasing levels of cAMP, a key second messenger. Conversely, phosphodiesterase breaks down cAMP.

-Insulin enhances phosphodiesterase activity in the liver, thereby reducing cAMP levels and influencing glycogen metabolism (Satyanarayana and Chakrapani, 2013).

- Glycogen synthesis is regulated by glycogen synthase, which exists in two forms : the active, non-phosphorylated form (glycogen synthase "a") and the inactive, phosphorylated form (glycogen synthase "b"). Phosphorylation of "a" to "b" is catalyzed by cAMP-dependent protein kinase, leading to inactivation. Conversely, protein phosphatase I can reconvert glycogen synthase "b" to the active "a" form, thus promoting glycogenesis (Satyanarayana and Chakrapani, 2013).

- Glycogen breakdown is regulated by hormones such as epinephrine and glucagon, which promote glycogenolysis via cAMP. cAMP activates protein kinase, which phosphorylates glycogen phosphorylase, converting it into its active form. This active kinase then phosphorylates inactive glycogen phosphorylase "b" to activate it into glycogen phosphorylase "a", which leads to glycogen degradation. Conversely, protein phosphatase I can inactivate glycogen phosphorylase "a" by dephosphorylizing it to reconstitute it into "b" (Satyanarayana and Chakrapani, 2013).

2.5. Regulation of the pentose phosphate pathway

-The pathway is primarily regulated by NADP+ levels. The first reaction catalyzed by G6PD is the rate-limiting step and is inhibited by NADPH.

-The oxidative phase is therefore controlled by NADP+ levels, and the non-oxidative phase by the need for pentose phosphates.

-Insulin will induce the G6PD enzyme and thus increase the overall pathway (Vasudevan et al., 2011).

2.6. Regulation of lipolysis

- Lipases in adipose tissue are activated by hormones such as epinephrine, norepinephrine, glucagon, and adrenocorticotropic hormone (ACTH).

- In adipose cells, these hormones trigger seven-transmembrane domain (7TM) receptors, which activate adenylate cyclase. The increased level of cyclic AMP then stimulates protein kinase A, which activates lipases by phosphorylating. Thus, epinephrine, norepinephrine, glucagon, and ACTH induce lipolysis. Conversely, insulin inhibits lipolysis (Berg et al., 2002).

2.7. Regulation of β-oxidation

-The availability of free fatty acids regulates net utilization by beta-oxidation.

-Fatty acid levels, are controlled by the glucagon:insulin ratio. Glucagon increases free fatty acid levels, while insulin has the opposite effect.

-CAT-I regulates fatty acid uptake into mitochondria. Malonyl CoA inhibits CAT-I activity. Thus, during de novo fatty acid synthesis, β-oxidation is inhibited (Vasudevan et al., 2011).

2.8. Regulation of lipogenesis

2.8.1. Allosteric regulation

-Acetyl CoA carboxylase : Citrate activates this enzyme, while palmitoyl CoA and malonyl CoA inhibit it. Citrate levels are only high when both acetyl CoA and ATP are abundant (Vasudevan et al., 2011; Satyanarayana and Chakrapani, 2013).

2.8.2. Covalent modification

Covalent modification is another regulatory mechanism. Phosphorylation inactivates acetyl CoA carboxylase (Vasudevan et al., 2011).

2.8.3. Hormonal regulation

- Insulin stimulates lipid synthesis (Kersten, 2001; Saponaro et al., 2015).
- Glucagon and catecholamines inhibit fatty acid synthesis (Saponaro et al., 2015).
- Growth hormones reduce lipogenesis in adipose tissue (Kersten, 2001).
- Leptin inhibits lipogenesis (Kersten, 2001).

2.9. Regulation of ketogenesis

-Ketogenesis is controlled by three enzymes : hormone-sensitive lipase, acetyl-CoA carboxylase, and HMGCS2.

- Insulin inhibits ketogenesis by inhibiting hormone-sensitive lipase, suppressing HMGCS2 expression in the liver, and limiting substrate availability by reducing lipolysis in adipose tissue and stimulating acetyl-CoA carboxylase.

- Glucagon stimulates ketogenesis by activating hormone-sensitive lipase and promoting HMGCS2 expression via peroxisome proliferator-activated receptor alpha (PPARα), thereby increasing the ketogenic flux of fatty acids.

-Other hormones, such as epinephrine and noradrenaline, also activate ketogenesis by stimulating lipolysis (Leonard, 2020; Mooli and Ramakrishnan, 2022).

2.10. Regulation of amino acid metabolism

-Glutamate dehydrogenase (GDH) is a zinc-containing enzyme. Its activity is controlled allosterically : ATP and GTP inhibit GDH**,** while ADP and GDP activate it.

-Steroid and thyroid hormones reduce GDH activity.

CHAPTER 3 : STUDY OF BIOCHEMICAL TECHNIQUES

1. Electrophoresis

1.1. Definition and principle

Electrophoresis is a technique used to separate charged particles such as ions, molecules, or macromolecules based on their movement in an electric field within a liquid medium. Any electrically charged particle in an aqueous solution migrates toward the electrode with the opposite charge. The speed of migration depends on the particle's charge and the applied voltage (directly proportional) and its size (inversely proportional). All molecules of different sizes and/or charges can be separated in this way. Electrophoretic analysis can, in principle, be applied to all charged particles under given experimental conditions, such as small cations or anions, organic acids, amino acids, peptides, saccharides, lipids, proteins, nucleotides, nucleic acids, and even entire subcellular particles or whole cells. In practice, proteins and nucleic acids are the most frequently used objects for electrophoretic separation (Plášteník, 2009).

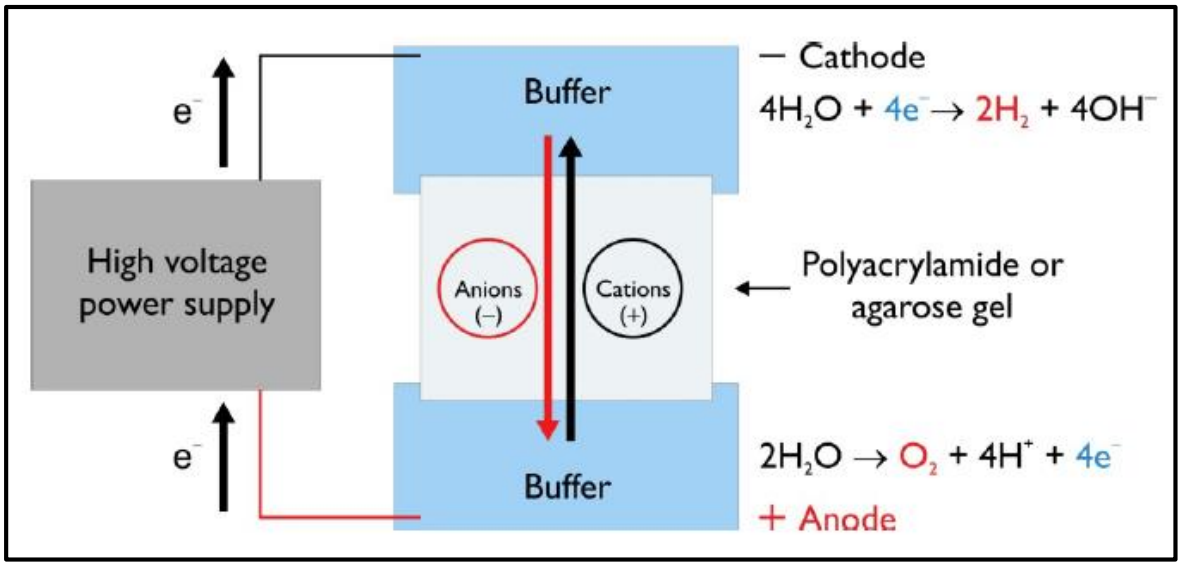

Figure 37: The principle of electrophoresis (Pál, 2013).

1.2. Components

***Buffer :** Conducts the electric current and maintains the pH of the medium.

***Wicks :** Connect the support medium to the buffer to complete the circuit.

***Support :** Serves as a matrix for the separation of molecules.

***Cover :** Reduces buffer evaporation and prevents contamination during electrophoresis.

***Power supply :** Provides an electric field for the movement of charged particles.

***Densitometer :** The quantification of the separated bands is carried out by comparing their optical density (Sonagra et al., 2025).

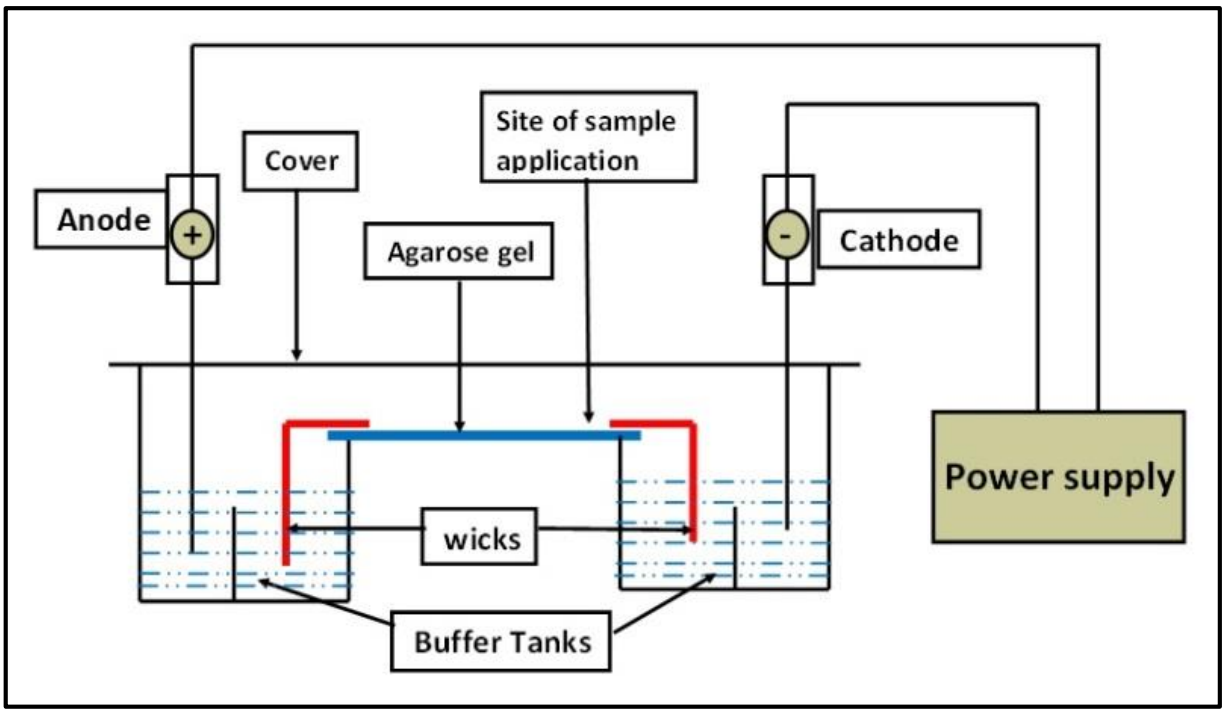

Figure 38: Diagram of electrophoresis apparatus (Sonagra et al., 2025).

1.3. Types

1.3.1. Liquid vein electrophoresis

Electrophoretic techniques classically use a solid support (paper, cellulose acetate, polyacrylamide gel, etc.). However, electrophoresis was originally described in a form free of any support, vein electrophoresis, but this technique with mediocre performance was quickly superseded. It was only in the 1970s and 1980s that several teams taking up this approach showed that it is possible to carry out electrophoretic separation in a liquid stream (i.e. in a non-gelled medium), using a tube with a small internal diameter (< 200 µm) (Feuilloley et al., 1999). Migration is mainly disrupted by convection and diffusion phenomena. In addition, since the sample is deposited uniformly in the vein at the beginning of migration, separation is not optimal (Vigneron, 1984).

1.3.2. Zone electrophoresis

Most practical applications of electrophoresis in biochemistry use a form of zone electrophoresis, in which the aqueous ionic solution is contained within a solid support and samples are applied as spots or bands of material. Paper electrophoresis, cellulose acetate electrophoresis, and gel electrophoresis are all examples of zone electrophoresis (Switzer and Garrity, 1999).

*** Paper electrophoresis :** Paper electrophoresis is a commonly used electrophoretic method for the analysis and separation of small molecules. This method is not used to separate

macromolecules (e.g., proteins) because the adsorption and surface tension associated with paper electrophoresis generally alter or denature macromolecules, resulting in poor resolution (Switzer and Garrity, 1999).

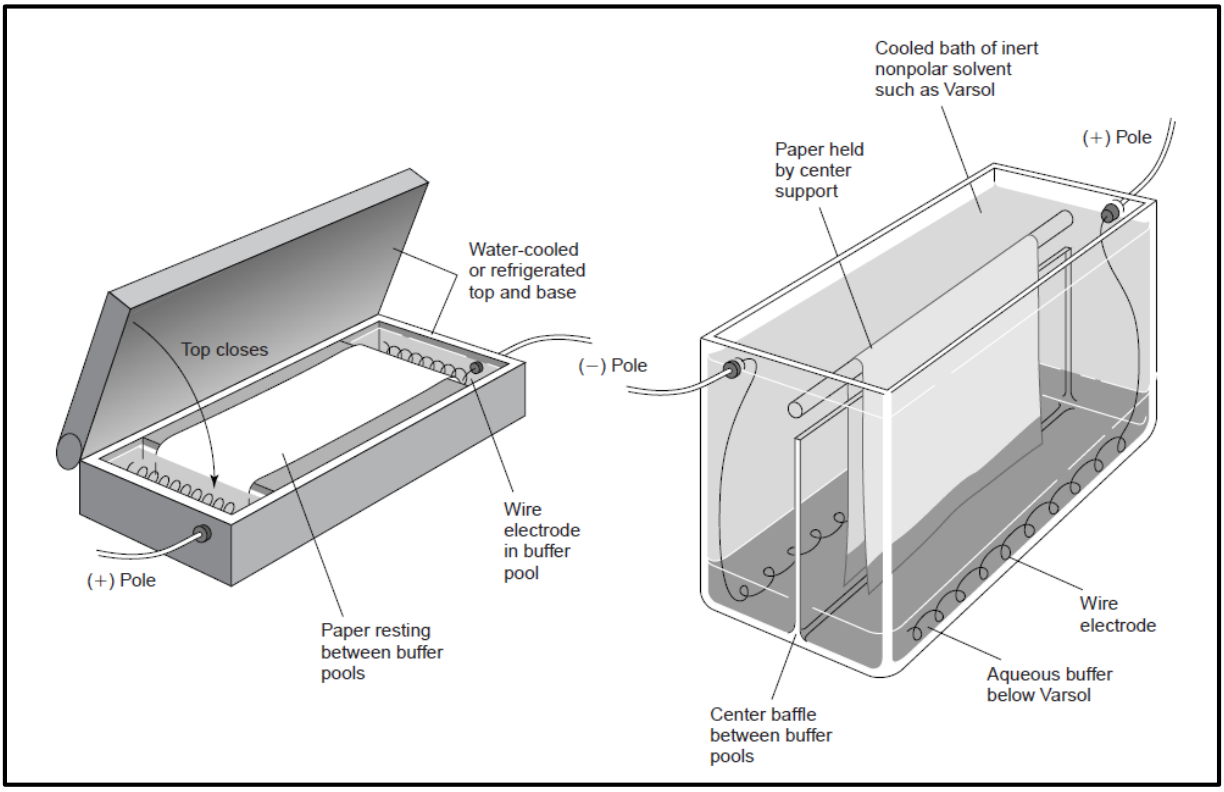

Figure 39: Paper electrophoresis (Switzer and Garrity, 1999).

***Cellulose acetate electrophoresis :** Cellulose acetate electrophoresis is notably used in the clinical analysis of serum samples. Compared to paper, cellulose acetate has the advantage of being a much more homogeneous medium, with uniformly sized pores, and it does not adsorb proteins as paper does. Therefore, there are far fewer protein streaks and the resolution is better, although still not equal to that obtained with polyacrylamide gels. The method is, however, much simpler to set up and perform. Individual samples are generally analyzed on cellulose acetate strips (2.5 cm to 12 cm), although multiple samples are frequently analyzed on larger sheets (Walker, 2010).

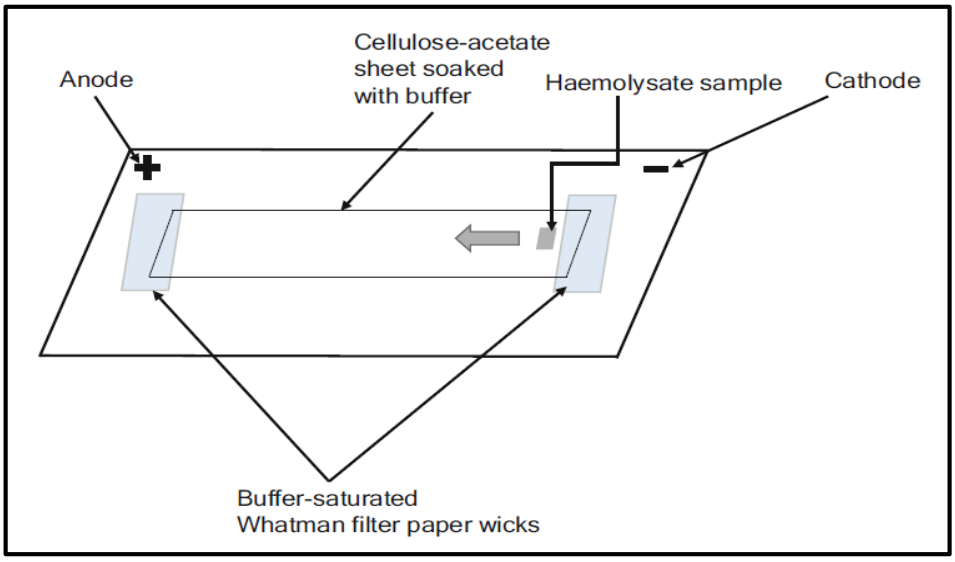

Figure 40: Diagram of cellulose acetate electrophoresis (Kumar and Derbigny, 2019).

*** Agarose gel electrophoresis :** Agarose gel electrophoresis is a widely used technique in molecular biology for separating DNA and RNA fragments according to their size. It uses an agarose gel matrix and an electric field to direct negatively charged nucleic acids towards the positive electrode (anode) (Kaja et al., 2025).

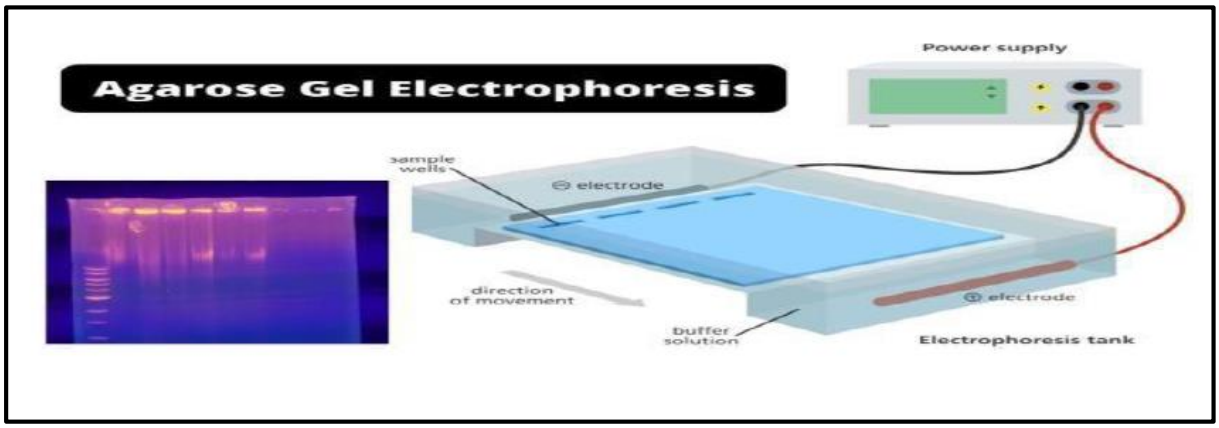

Figure 41: Agarose gel electrophoresis (Kaja et al., 2025).

*** Polyacrylamide gel electrophoresis :** Acrylamide gel electrophoresis is often referred to as PAGE, short for polyacrylamide gel electrophoresis (Walker, 2010). Polyacrylamide gels can be used for the separation and analysis of proteins and small nucleic acid molecules. The resolution of the PAGE method is so high that, in the size range of approximately 10 to 1,000 nucleotide units, it is capable of separating DNA molecules that differ in length by only a single monomer unit (Pál, 2013).

*** SDS-PAGE :** SDS-PAGE is the most widely used method for the qualitative analysis of protein mixtures. It is particularly useful for monitoring protein purification and, because it is

based on separating proteins according to their size, it also allows for the determination of their relative molecular weight (Walker, 2010).

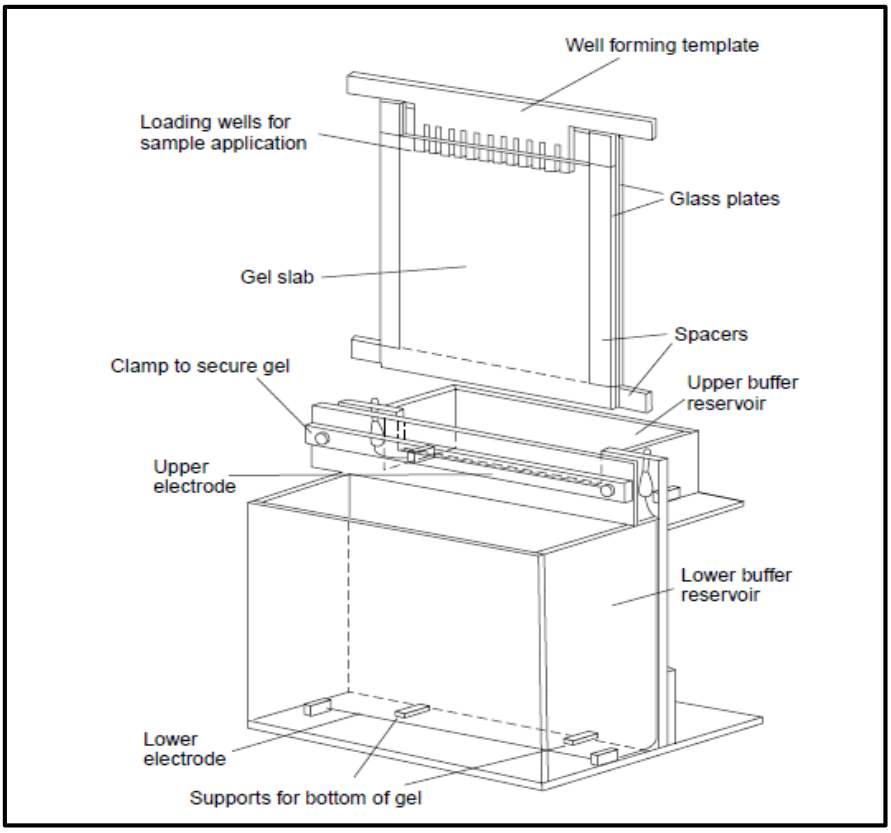

Figure 42: Typical device for SDS-PAGE on vertical plate (Wilson and Walker; 1994).

*** Two-dimensional polyacrylamide gel electrophoresis :** Two-dimensional polyacrylamide gel electrophoresis combines the polyacrylamide gel electrophoresis (IEF) technique (first dimension), which separates proteins in a mixture according to their charge (pI), and the size separation technique of SDS-PAGE (second dimension). The combination of these two techniques to obtain two-dimensional (2D) polyacrylamide gel electrophoresis offers a highly sophisticated analytical method for the analysis of protein mixtures (Walker, 2010).

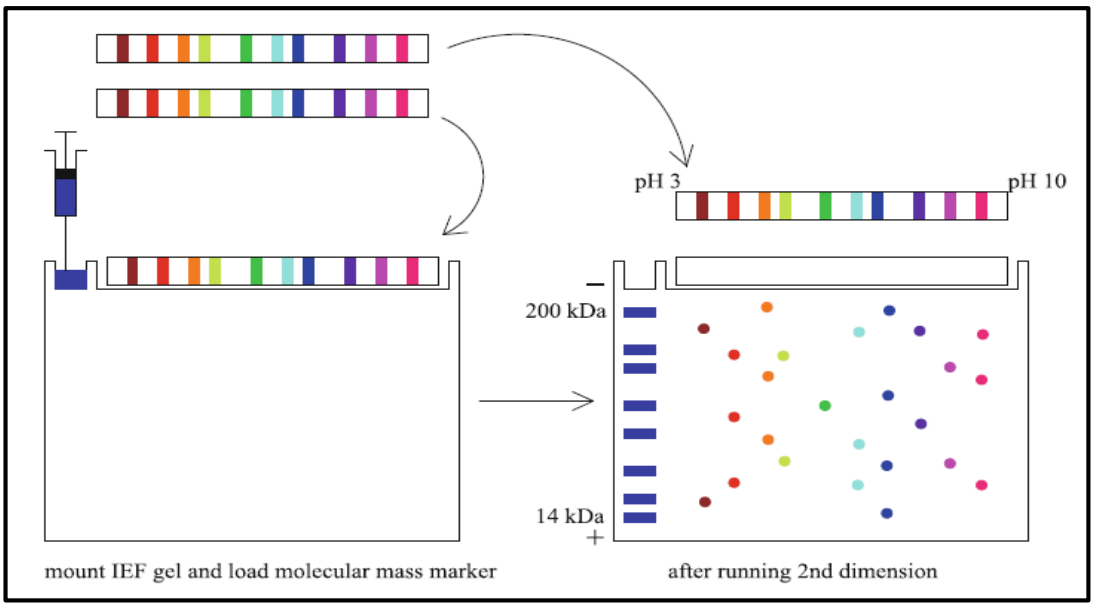

Figure 43: Two-dimensional electrophoresis (Buxbaum, 2025).

*** Isoelectric electrophoresis (Electrofocusing) :** This method is ideal for separating amphoteric substances such as proteins, as it relies on separating molecules according to their different isoelectric points. This method offers high resolution, allowing the separation of proteins whose isoelectric points differ by only 0.01 pH units (Walker, 2010).

1.3.3. Capillary electrophoresis

Capillary electrophoresis allows the separation of a wide spectrum of biological molecules, including amino acids, peptides, proteins, DNA fragments (e.g., synthetic oligonucleotides) and nucleic acids, as well as many small organic molecules such as drugs or even metal ions (Walker, 2010).

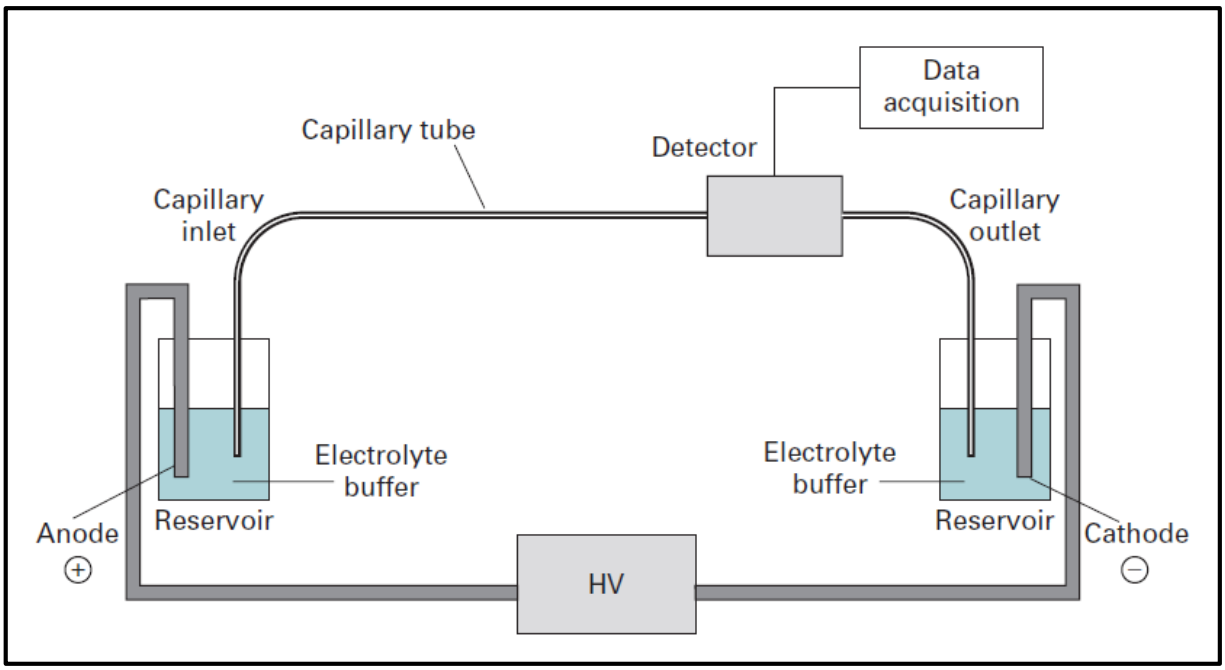

Figure 44: Diagram of a capillary electrophoresis apparatus (Walker, 2010).

2. Chromatography

2.1. Definition

Chromatography is a method used in the laboratory to separate mixtures. The mixture is dispersed in a liquid called the mobile phase, which carries it through an apparatus containing a different substance called the stationary phase. The components of the mixture separate because they move at different speeds. Separation is achieved through differential partitioning between the mobile and stationary phases. Differences in retention on the stationary phase, due to small variations in the partition coefficient of a compound, impact the separation (Rushikesh et al., 2024).

2.2. Principle

The foundation of chromatography rests on the idea that mixtures of molecules applied to solid and fluid surfaces (stationary, stable phases) separate from one another by moving with the help of a mobile phase. Molecular characteristics related to adsorption (liquid-solid), partitioning (liquid-solid), and affinity (or differences in molecular weights) are the factors that influence this separation process. These variations cause some combined components to remain longer in the stationary phase and to pass through the chromatographic system more slowly, while others pass through it more quickly.

Three components therefore form the basis of the chromatography technique :

* **Stationary phase :** This phase is always composed of a solid phase or a layer of liquid adsorbed on the surface of a solid support.

* **Mobile phase :** This phase is always composed of a liquid or a gas.

* **Separated molecules :** The type of interaction between the stationary phase, the mobile phase and the substances contained in the mixture is the fundamental component that allows the separation of molecules (Rushikesh et al., 2024).

2.3. Types

2.3.1. Column chromatography

Column chromatography is the most widely used technique for the purification of biomolecules. The sample to be separated is first deposited onto a column (stationary phase), followed by the washing buffer (mobile phase). Their flow through the column material, which is supported by a fiberglass mat, is ensured. The samples accumulate at the bottom of the apparatus depending on time and volume (Coskun, 2016).

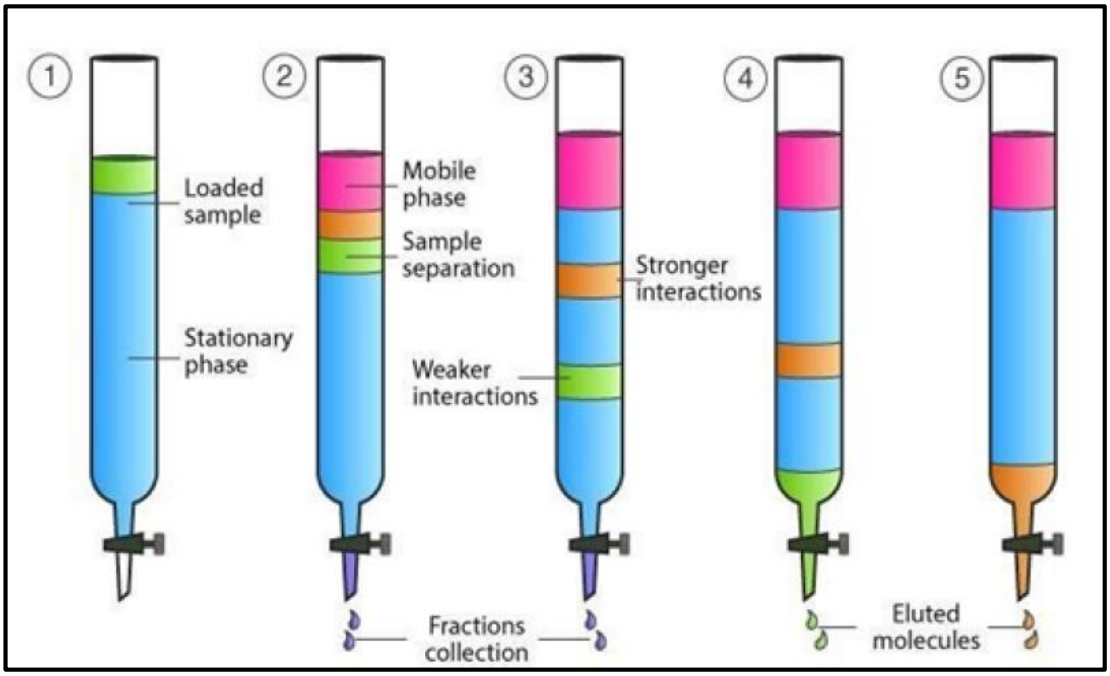

Figure 45: Column chromatography (Rushikesh et al., 2024).

2.3.2. Ion-exchange chromatography

Ion exchange is probably the most classic and widespread type of chromatography in biochemistry. It relies on the differential electrostatic affinities of molecules with a surface charge for an inert and charged stationary phase or a solid support. There are two types of ion-exchange chromatography, determined by the charges present on both the stationary phase and the molecules that will be adsorbed onto it. Cation exchange occurs when the stationary phase carries a negative charge. It has an affinity for molecules in solution with a positive surface charge (i.e., cations). The opposite is true for anion exchange: the stationary phase carries a

positive charge that has an affinity for molecules with a negative surface charge (i.e., anions) (Switzer, 1999).

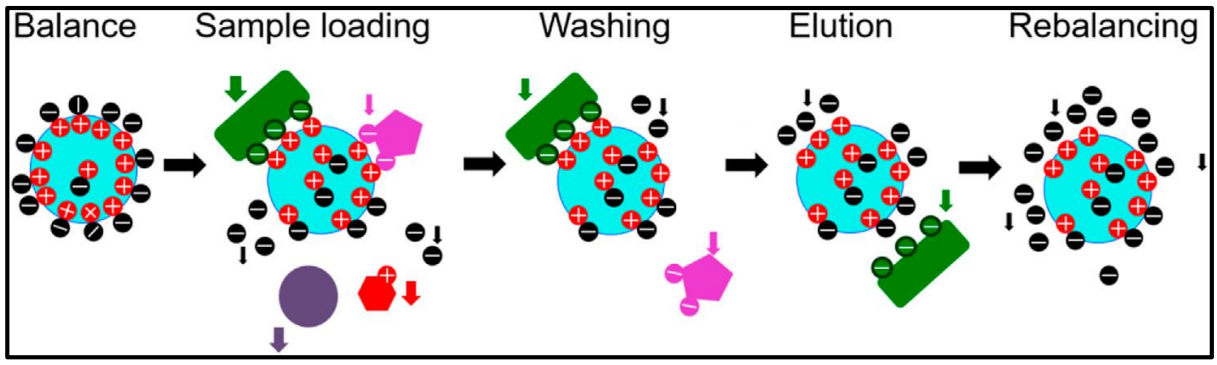

Figure 46: Principle of ion exchange chromatography (Du et al., 2022).

2.3.3. Affinity chromatography

Affinity chromatography enables highly selective separation of biomolecules through their specific interactions. This separation technique is unique because it relies on the biological function or unique chemical structure of a given biomolecule. In affinity chromatography, the biomolecule's interaction partner is immobilized on a chromatographic resin. This ligand, attached to the stationary phase, reversibly binds the desired biomolecule to the multicomponent mobile phase. Materials can be eluted from the column by changing the composition of the mobile phase. This technique offers high selectivity, high resolution, and generally high capacity for the desired protein. The degree of purification can reach thousands of times, and the yield obtained is generally very good (Hegyi and Kovács, 2013).

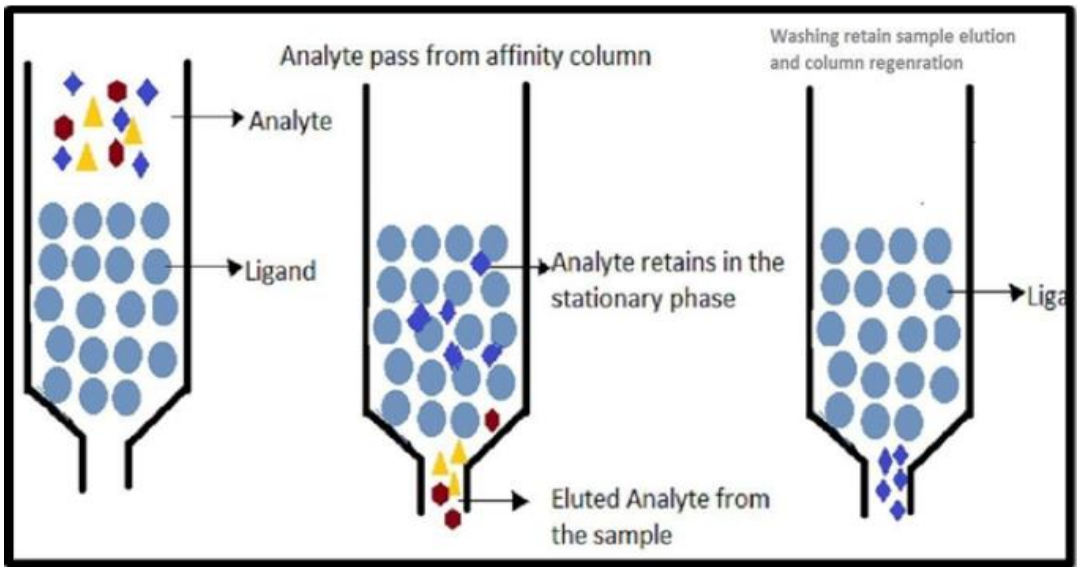

Figure 47: Schematic of affinity chromatography (Rushikesh et al., 2024).

2.3.4. Gas chromatography

In this method, the stationary phase is a column placed in the instrument and containing a liquid stationary phase adsorbed onto the surface of an inert solid. Gas chromatography is a gas-liquid chromatography technique. Its carrier phase consists of gases such as helium (He) or nitrogen (N_2). The mobile phase, an inert gas, flows through a column under high pressure. The sample to be analyzed is vaporized and passes into the gaseous mobile phase. The components of the sample are dispersed between the mobile and stationary phases on the solid support. Gas chromatography is a simple, versatile, highly sensitive, and rapid technique that allows for extremely precise separation of very small molecules. It is used for the separation of very small quantities of analytes (Coskun, 2016).

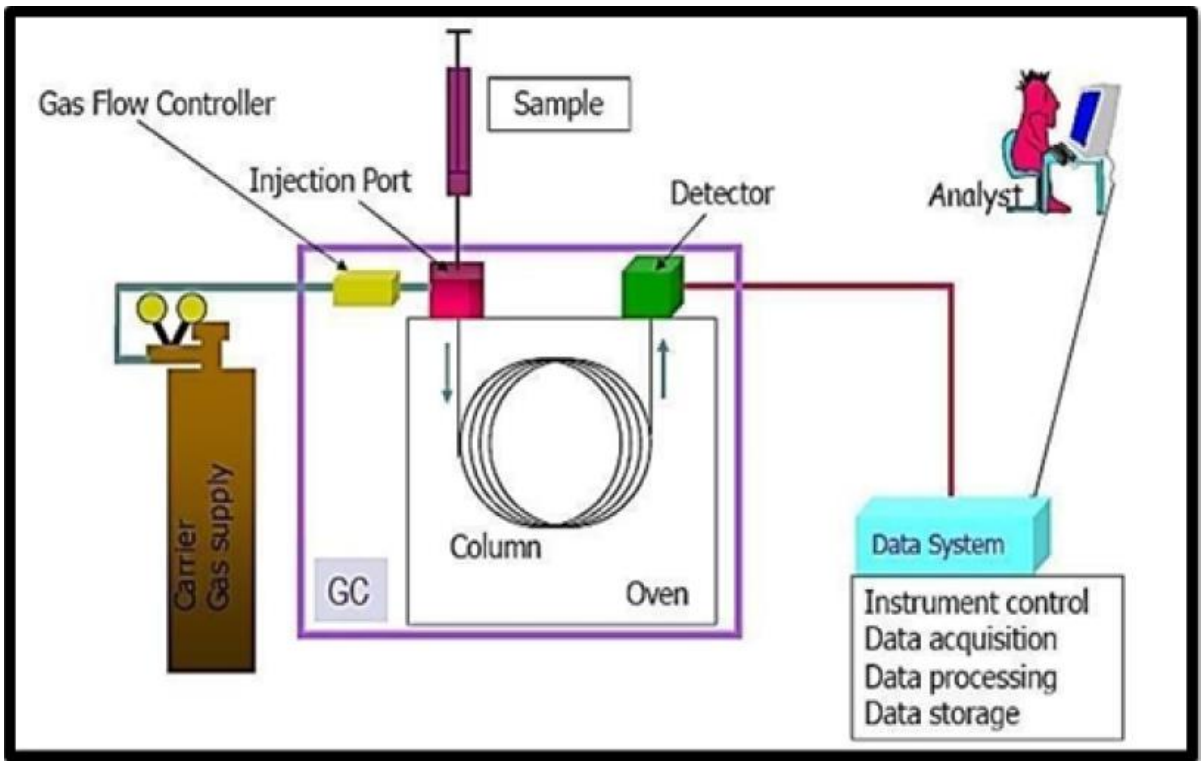

Figure 48: Diagram of gas chromatography (Rushikesh et al., 2024).

2.3.5. High-performance liquid chromatography (HPLC)

The analytical chemistry method of high-performance liquid chromatography (HPLC), formerly known as high-pressure liquid chromatography, allows for the separation, identification, and quantification of each component of a mixture. It uses pumps to move a column of solid adsorbent material through a pressurized liquid solvent containing the sample combination.

The adsorbent material and each component of the sample interact slightly differently, resulting in variable flow rates for the different components and their separation at the column outlet (Rushikesh et al., 2024).

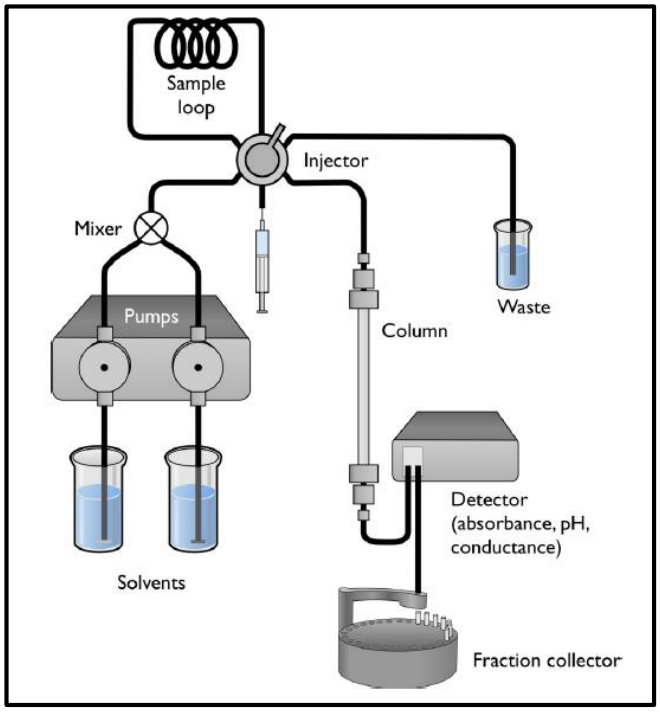

Figure 49: Diagram of HPLC (Hegyi et Kovács, 2013).

2.3.6. Paper chromatography

In paper chromatography, the support consists of a layer of cellulose highly saturated with water. In this method, a thick filter paper serves as the support, and water droplets deposit in its pores to form the stationary liquid phase. The mobile phase consists of a suitable fluid placed in a developing tank. Paper chromatography is a liquid-liquid chromatography technique (Coskun, 2016).

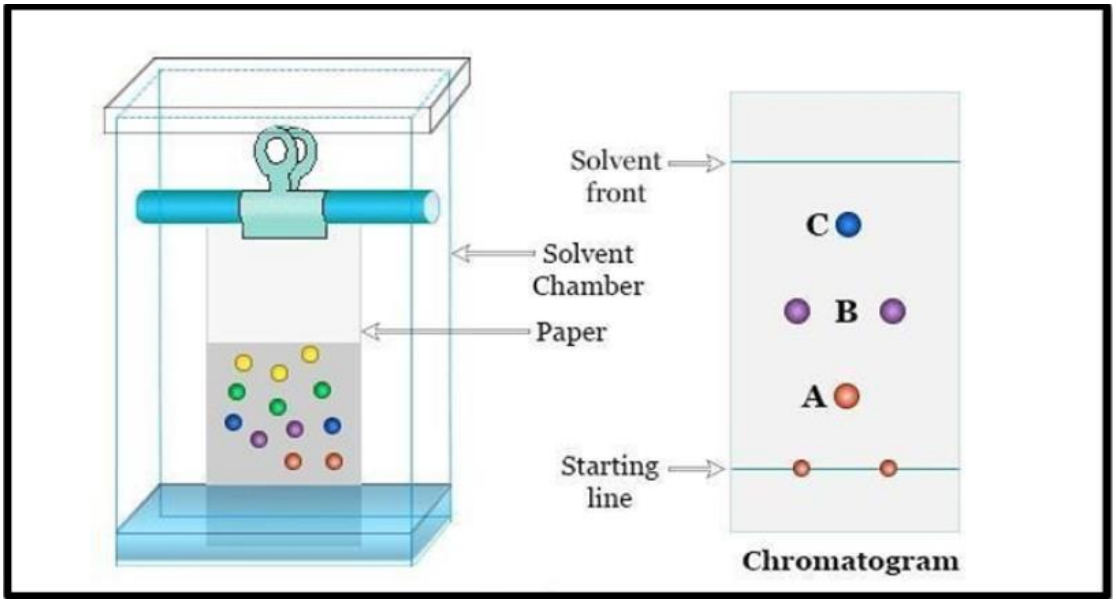

Figure 50: Diagram of paper chromatography (Rushikesh et al., 2024).

3. Centrifugation

3.1. Definition

Centrifugation can be defined as a separation method. It separates the particles of a sample according to their shape, size and density through the centrifugal force generated by the rotation (Sonmez et al., 2021).

3.2. Principle

Centrifugation allows the separation of all types of particles based on their sedimentation properties. These properties depend on several factors, including size, density, and shape. However, density and shape vary considerably depending on the composition of the solution in which the particles are suspended. Particles are separated primarily based on their density or size; in the latter case, density affects sedimentation, but, in most cases, to a much lesser extent than size.

In centrifugation, it is important to differentiate between centrifugation speed (revolutions per minute, rpm) and relative centrifugal force (RCF or G), as these two values are often confused. The centrifugal force generated by a centrifuge can be easily calculated from the following equation:

$$\mathbf{RCF = 11.18 \times R \times (RPM/1000)^2}$$

Where R is the distance to the center of rotation in centimeters; in other words, the centrifugal force increases as the particles descend in the centrifuge tube. As a general rule, the higher the centrifugal force, the shorter the separation time (Rickwood, 2001).

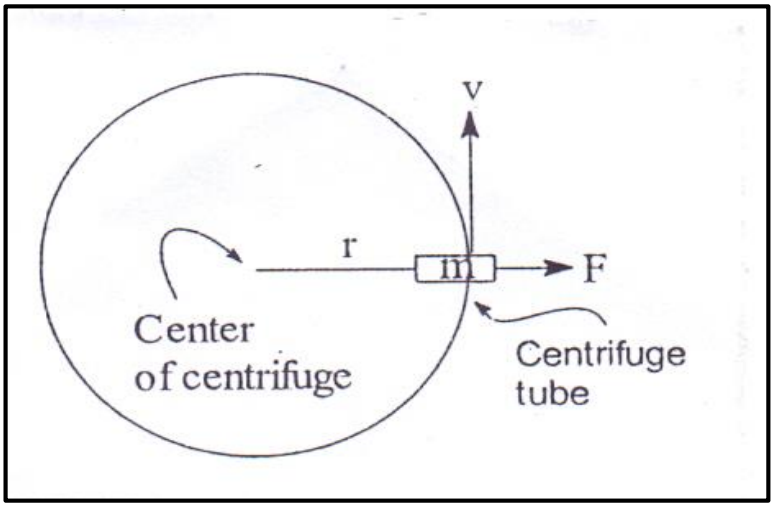

Figure 51: Illustration of the principle of centrifugation. F = centrifugal force, m = mass of the body, v = velocity of the body, r = radius of the circle of rotation (Majekodunmi, 2015).

3.3. Equipment

3.3.1. Centrifuges

A centrifuge is a device designed for separating mixtures. It is therefore commonly used in medical laboratories to prepare serum, plasma, and urine samples for analysis (Sonmez et al., 2021).

3.3.1.1. Clinical desktop centrifuges

Desktop clinical centrifuges are the simplest and least expensive centrifuges currently available. These centrifuges are used for substances that can sediment rapidly (yeast cells, blood cells, large precipitates from certain reactions) or for fractionating small quantities of these substances. They can operate at a maximum speed of 3,000 rpm at room temperature (Gandhi et al., 2022).

3.3.1.2. High-speed centrifuges

High-speed centrifuges can reach speeds of 20,000 to 25,000 rpm. They are equipped with a cooling system to prevent overheating and a braking device to slow the rotor after centrifugation. They are used to separate cells, cellular debris, microorganisms, immune precipitates, ammonium sulfate precipitates, and cellular organelles such as lysosomes, mitochondria, and nuclei. However, they are not effective for sedimenting viruses or very small organelles (Gandhi et al., 2022).

3.3.1.3. Microcentrifuges

Microcentrifuges (microfuges) allow the separation of small quantities of samples (0.5 to 2 ml) at speeds of 12,000 to 13,000 rpm. They are used to isolate cell organelles, DNA, and to perform phenol extractions. They use smaller tubes, but adapters allow the use of larger tubes. Some microcentrifuges have temperature control for sensitive samples (Gandhi et al., 2022).

3.3.1.4. Ultracentrifuge

These ultra-high-speed centrifuges (up to 50,000 rpm) have broadened the scope of research by enabling the fractionation of subcellular organelles and macromolecules in their native form. They also allow the separation of smaller structures such as viruses, proteins, and ribosomes, while maintaining temperature through a refrigeration system (Gandhi et al., 2022).



Figure 52: Types of centrifuges. A = clinical desktop centrifuges, B = high-speed centrifuges, C = microcentrifuge, D = ultracentrifuge (Gandhi et al., 2022).

3.3.2. Rotors

3.3.2.1. Fixed-angle rotors

The tubes are positioned at an angle of 14 to 40° from the vertical. The particles move rapidly outwards and travel a short distance. These rotors are used for differential centrifugation. The acceleration and deceleration of the rotor automatically reorient the tube (Gandhi et al., 2022).

3.3.2.2. Vertical tube rotors

These rotors are held vertically parallel to the rotor axis. The time required for particle separation is shorter, as the particles tend to travel a shorter distance (Gandhi et al., 2022).

3.3.2.3. Swinging-bucket rotors

As the rotor accelerates, this type of rotor orients itself to a horizontal position, forcing the sample to travel a longer distance, thus improving separation, as in density gradient centrifugation. Supernatant removal is much easier because it does not disturb the pellet (Gandhi et al., 2022).

3.3. Types of centrifugation

3.3.1. Differential centrifugation

Differential centrifugation separates particles in a suspension according to their size and density : the largest and densest particles settle more quickly. By applying a series of increasing centrifugal forces to a homogenate, pellets are obtained containing successively smaller particles, such as nuclei, mitochondria, and lysosomes. This method is commonly used to harvest cells or produce crude subcellular fractions. However, it has limitations, including contamination and low yields, which can be partially corrected by washing and repeating the centrifugation (Majekodunmi, 2015).

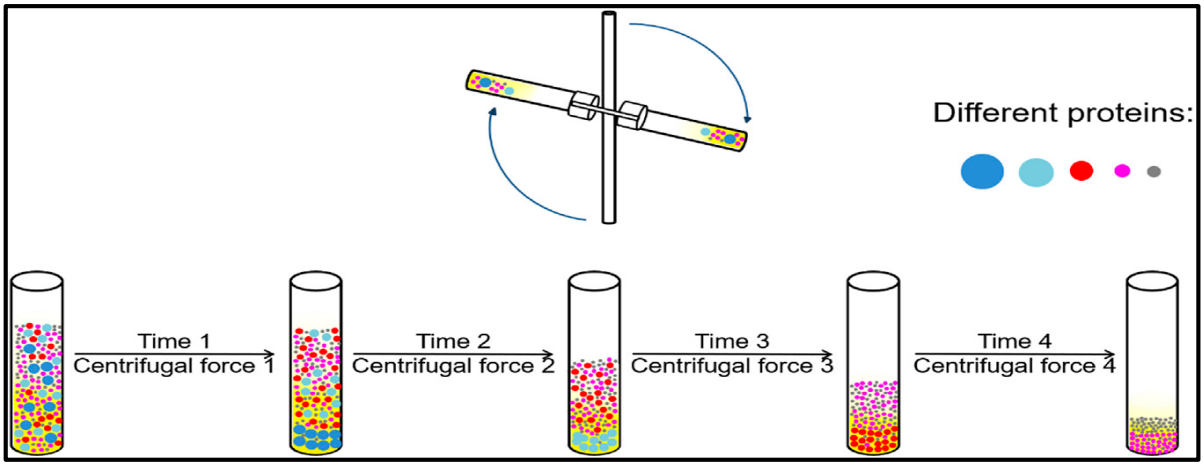

Figure 53: Differential centrifugation (Du et al., 2022).

3.3.2. Density gradient centrifugation

Density gradient centrifugation, or zonal centrifugation, involves depositing a sample onto the surface of a density gradient (often sucrose or cesium chloride). Under the effect of centrifugal force, the molecules separate according to their sedimentation rate, forming distinct zones. This technique offers high resolution and low cost, and is widely used to purify cells, organelles, viruses, bacteria, nucleic acids, and proteins. For example, it can be used to separate the inner and outer membranes of liver tissue (Du et al., 2022).

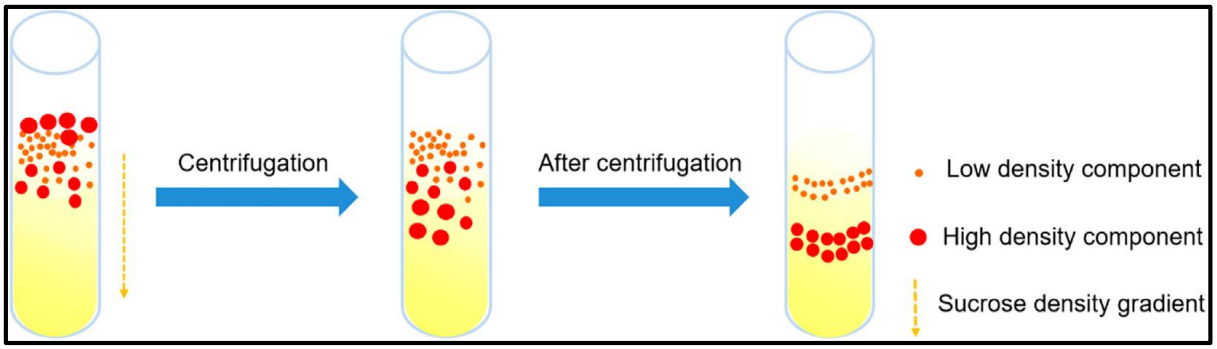

Figure 54: Density gradient centrifugation (Du et al., 2022).

4. Spectroscopy

4.1. Definition and principle

Spectrophotometry is a technique used to measure the amount of light absorbed by a solution. The basic principle involves passing a beam of light through a sample and measuring the intensity of the light reaching a detector. The amount of light absorbed by the sample is directly proportional to the concentration of the absorbing species, according to Beer-Lambert's law. This relationship can be used to determine the concentration of solutes in the solution by comparing the measured absorbance to a standard curve created from known concentrations (Nishchala et al., 2024).

4.2. Electromagnetic spectrum

The electromagnetic spectrum is composed of a continuum of waves with different properties. Figure 56 shows several regions of this spectrum, including X-rays (X-ray crystallography, up to 7 nm), ultraviolet (UV, 200-400 nm), visible (VIS, 400-700 nm), infrared (IR, 1000-100,000 nm) and radio waves (NMR, 106-1010 nm), which are classified according to frequency and wavelength (Shakya et al., 2021).

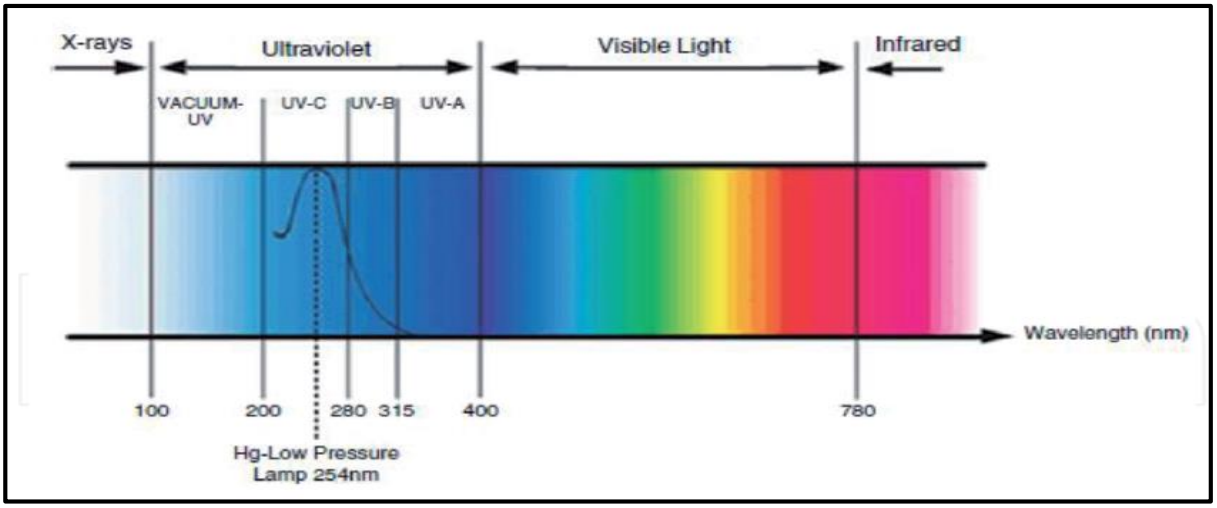

Figure 55: Electromagnetic spectrum (Murali and Mohd Y, 2022).

4.3. Transmission and absorbance

A spectrometer produces a variety of wavelengths because different compounds absorb better at different wavelengths. Once the intensity of the light is known after it passes through the cuvette, it can be related to the transmittance (T). Transmittance is the fraction of light that passes through the sample. It can be calculated using the following equation :

Transmittance : $T = I_t/I_0$

I_t is the light intensity after the light beam has passed through the basin and Io is the light intensity before the light beam passes through the basin (Renjini and Dani, 2017).

When light passes through or is reflected by a sample, the amount of light absorbed is the difference between the incident radiation (Io) and the transmitted radiation (I). The amount of light absorbed is expressed as absorbance (A) (Agilent, 2021).

Absorbance : $A= -\log (T)$

4.4. Beer-Lambert law

Quantitative measurements in spectrophotometry are based on Beer-Lambert's law of light absorption by solutions.

From the degree of absorption, the concentration of the absorbing component can be deduced using the following equation. If the intensity of light, I_0, decreases to I_t after traveling a path of length L in a medium, then, according to Beer-Lambert's law: (Hegyi et al., 2001).

$$\mathbf{A= \varepsilon.c.l}$$

A : absorbance, ε : in (mol-1.L.cm-1) the molar extinction coefficient of the absorbing species in solution, c : (in mol.L-1) the concentration of the absorbent species, l : (in cm) the length of the optical path.

4.5. Instrumentation

Spectrophotometers are instruments that use monochromators to select the wavelength. When the instrument's transmittance is the ratio of the two radiant powers, the instrument is called a spectrophotometer (Gandhi et al., 2022). The main components of a spectrophotometer are:

*a monochromatic light beam generator comprising one or two radiation sources coupled to a single or double grating monochromator;

*an optical system that shapes the beam and allows operation in "single beam" or "double beam" mode;

*a device for positioning the object to be measured;

*a detection system consisting of one or more detectors (Dubard et al., 2016).

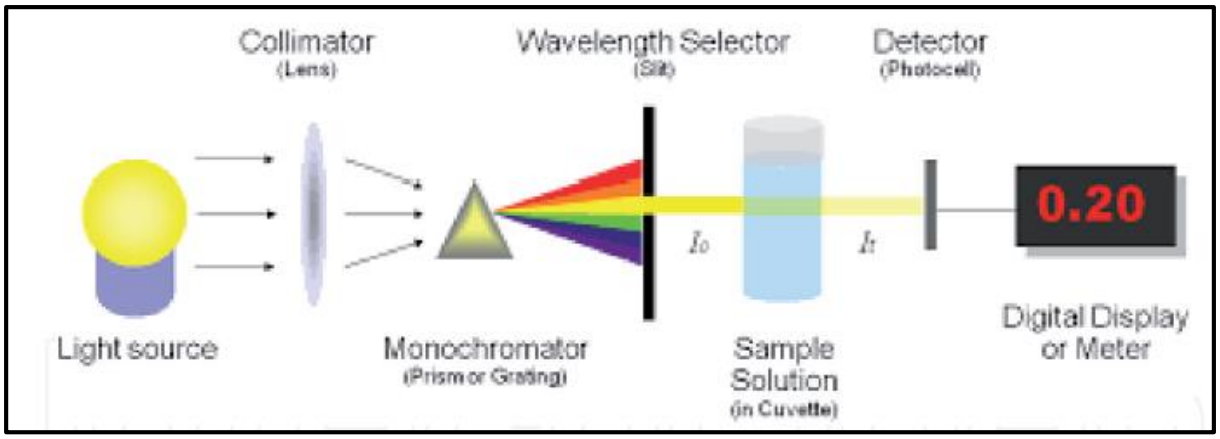

Figure 56: Basic instrumentation of the spectrophotometer (Dadi and Yasir, 2022).

4.6. Types

4.6.1. UV-Visible spectroscopy

4.6.1.1. Principle

UV-visible spectroscopy is a powerful analytical technique used to measure the absorbance of light by a sample in the ultraviolet (UV) and visible (Vis) regions of the electromagnetic

spectrum (Dubey et al., 2024). The absorption law is the basic principle of UV-visible spectrophotometry. This law describes the relationship between the thickness of the absorbing material and the concentration of the sample (Dadi and Yasir, 2022).

4.6.1.2. Instrumentation

***Light source :** Hydrogen and deuterium lamps are used as UV light sources, while for the visible range, tungsten filament lamps are most commonly used.

***Sample holders :** Cuvettes are used as sample holders in both the UV and visible ranges. These cuvettes are made of quartz or ordinary glass. Quartz or silica cuvettes are generally used in the UV region, while glass cuvettes are used in the visible region. Most cuvettes have a standard path length of 1 cm.

***Monochromators :** A monochromator converts polychromatic radiation into monochromatic radiation, whereby the wavelengths of this radiation are translated into very narrow bands.

***Detectors :** Photovoltaic cells, phototubes, and photomultiplier tubes are commonly used detectors in the UV and visible ranges (Dadi and Yasir, 2022).

4.6.2. Infrared spectroscopy

4.6.2.1. Principle

When a molecule absorbs light with a wavelength longer than that of UV and visible light, vibrational transitions can occur within the molecule. These vibrational transitions lead to the formation of an IR spectrum. These vibrational transitions are due to the occurrence of electronic transitions when a given substance absorbs light energy (Dadi and Yasir, 2022).

4.6.2.2. Instrumentation

***Light source :** Xenon and tungsten lamps are generally used as light sources in the near-infrared region.

***Sample holders :** In the infrared region, quartz cuvettes are generally used as sample holders.

***Monochromator :** Gratings are used as monochromators in the infrared region.

***Detectors :** Detectors commonly used in IR spectrophotometry are indium gallium arsenide (InGaAs) semiconductor materials (Dadi and Yasir, 2022).

4.6.3. Spectrofluorometry

4.6.3.1. Principle

Fluorescence is the phenomenon in which a molecule absorbs light and then emits light of a longer wavelength. After absorbing radiation, the molecule moves to an excited energy level. It then returns to its ground state either in one step emitting light close to the absorbed wavelength or in several steps, emitting light of progressively longer wavelengths. These emissions produce the fluorescence spectrum**.** Fluorescence occurs very quickly (in about 10^{-7} seconds or less). Fluorometry also follows Beer-Lambert's law in its operating principle (Dadi and Yasir, 2022).

4.6.3.2. Instrumentation

The instrumentation of a spectrofluorometer differs from that of a spectrophotometer in two important aspects, in addition to other minor variations.

*There are two monochromators instead of one, as in a spectrophotometer ; one monochromator is placed before the sample holder and one after it.

*Fluorescence is maximal between 25 and 30°C, the sample holder is equipped with a device to maintain the temperature (Dadi and Yasir, 2022).

4.6.4. Atomic absorption spectroscopy (AAS)

4.6.4.1. Principle

When the molecules of the sample are subjected to volatilization, the atoms produced absorb a certain wavelength of light from a source which produces an atomic spectrum characteristic of the molecule (Dadi and Yasir, 2022).

4.6.4.2. Instrumentation

***Atomizers :** The most commonly used atomizers in AAS are electrothermal atomizers (ETAs). They are involved in converting sample molecules into individual gaseous atoms that can absorb light.

***Light source :** Typically, a hollow cathode lamp is used as the light source in AAS to produce a specific wavelength of light that is ideally absorbed by gaseous atoms. Nowadays, instruments with single- and dual-beam optics are available.

***Detectors :** For isolating and quantifying wavelengths of interest, and for controlling instrument operation and collecting and processing data, a computer system is used (Dadi and Yasir, 2022).

4.6.5. Nuclear magnetic resonance (NMR) spectroscopy

4.6.5.1. Principle

Nuclear magnetic resonance (NMR) is one of the most useful and powerful techniques for determining molecular structure. The principle of NMR is that when a strong magnetic field and a radio frequency emitter are applied to sample molecules, the atomic nuclei of these molecules are excited and form spectral lines in the spectrum (Dadi and Yasir, 2022).

4.6.5.2. Instrumentation

***Radiation source :** a radio frequency (RF) transmitter, which generates the radio frequency current. This current is delivered to the transmitting coil, which creates a signal used to excite protons in the magnetic field.

***A superconducting magnet :** which produces a magnetic field in the central volume of the magnet. The magnetic field produced varies from 1 to 10 T.

Advanced NMR spectrometers consist of superconducting solenoids that generate a magnetic field greater than 3.5 T. The magnet has a bore to hold the sample probe and a set of room temperature tuning coils (RTS) to reduce the inhomogeneity of the magnetic field over the active sample volume.

***A receiver :** receives the absorbed signal in a digitized form, generally called free induction decay (FID).

***A computer**

***An amplifier**

***An analog-to-digital converter (ADC)** (Dadi and Yasir, 2022).

5. PCR (polymerase chain reaction)

5.1. Definition

PCR is a simple enzymatic test that amplifies a specific DNA fragment from a complex pool of DNA. Dr. Kary Mullis, who discovered PCR, stated that it "allows you to select the DNA fragment you are interested in and obtain as much of it as you want." PCR can be performed using source DNA from a variety of tissues and organisms, including peripheral blood, skin, hair, saliva, and microbes (Garibyan and Avashia, 2013).

5.2. Principle

PCR allows for the exponential amplification of a specific DNA fragment from a sample, whether genomic, complementary (cDNA), or mitochondrial. This amplification relies on three successive steps: denaturation of the DNA strands, hybridization of the primers to the target sequence, and elongation of the new strands by Taq polymerase. The reaction takes place in a mixture containing template DNA, primers, Taq polymerase, and the four deoxyribonucleoside triphosphates (dNTPs) in excess in a buffer solution, placed in a thermocycler that precisely controls the temperature cycles, thus enabling the rapid production of large quantities of a specific DNA sequence (Kadri, 2019).

5.3. PCR Element

5.3.1. Template DNA

The double-stranded DNA molecule amplified by PCR is called the target DNA or template. The template defines the sequence into which new nucleotides are added during the PCR process (Shahzad et al., 2020).

5.3.2. Primers

Primers are single-stranded DNA molecules, usually commercially synthesized. They are polynucleotides of varying lengths. These short strands of polynucleotide DNA have a free 3' hydroxyl group, also called the 3' end. This free 3' hydroxyl group is required by DNA polymerase to add new nucleotides during the polymerization process, thus synthesizing a new complementary strand. To perform PCR, two primers are needed to amplify the two template strands: one primer for one strand (or sense strand), called the "forward primer," which forms the beginning of the template, and another primer for the complementary strand (or antisense strand), called the "reverse primer." Thus, the two primers bind to the 5' ends of the sense and antisense strands (Shahzad et al., 2020).

5.3.3. DNA polymerase

DNA polymerase begins synthesizing new DNA from the 3' end of the template. Primers bind to this end and are then elongated by the DNA polymerase. The most commonly used DNA polymerase is Taq, isolated from Thermus aquaticus, a thermophilic bacterium (Shahzad et al., 2020).

5.3.4. Nucleotides

PCR requires four different deoxynucleoside triphosphates (dNTPs) to synthesize new DNA strands : adenine (A), guanine (G), cytosine (C) and thymine (T) (Shahzad et al., 2020).

5.3.5. Buffer

The function of a PCR buffer solution is to provide the appropriate conditions and chemicals for DNA polymerase to achieve optimal activity and stability. Buffers often contain Tris-HCl, KCl, and sometimes $MgCl_2$ (Shahzad et al., 2020).

5.4. Steps of PCR

5.4.1. Denaturation

This involves separating the two strands of DNA, achieved by raising the temperature. The first step takes place at 94°C, called the denaturation temperature. At this temperature, the template DNA, which serves as a template during replication, is denatured: hydrogen bonds cannot be maintained at temperatures above 80°C, and the double-stranded DNA is denatured into single-stranded DNA (Kadri, 2019).

5.4.2. Hybridization

The second step is hybridization. This is performed at a temperature generally between 40 and 70 °C, called the primer hybridization temperature. Lowering this temperature allows hydrogen bonds to reform and thus the complementary strands to hybridize. Primers, short single-stranded sequences complementary to the regions flanking the DNA to be amplified, hybridize more readily than long-stranded template DNA (Kadri, 2019).

5.4.3. Elongation

The third step of PCR, called elongation, is performed at 72°C, the optimal temperature for Taq polymerase, which synthesizes the complementary strand from the primers. With each cycle, the new fragments serve as templates, allowing exponential amplification of the target sequence. Generally, 20 to 40 cycles are sufficient to obtain an analyzable amount of DNA, with each cycle theoretically doubling the amount of DNA present. A final elongation cycle is recommended for long sequences (>1 kilobase). PCR is rapid, lasting only 2 to 3 hours for 30 cycles, and can amplify DNA sequences up to 6 kilobases (Kadri, 2019).

CHAPTER 4 : USE OF ENZYMATIC AND IMMUNOLOGICAL TOOLS - STUDY OF PATHOPHYSIOLOGICAL VARIATIONS

1. Enzymatic tools

Enzyme methods are laboratory techniques used to measure enzyme activity. They are essential for studying enzyme kinetics and enzyme inhibition. Activity is monitored by measuring the decrease in substrates or the increase in products over a defined period. The assay is generally performed by determining enzyme activity with and without activation by an added coenzyme (Narayanan, 2021).

1.1. General information on enzyme activity

Enzymes are biological catalysts (also called biocatalysts) that accelerate biochemical reactions in living organisms. They can also be extracted from cells and used to catalyze a wide range of commercially important processes.

- As catalysts, enzymes are only required at very low concentrations and accelerate reactions without being consumed.

- Enzymes do not alter the equilibrium of a reaction. They do not fundamentally change the structure and energy of the products and reactants, but simply allow the reaction equilibrium to be reached more quickly.

- When an enzyme requires a cofactor for its activity, the inactive protein component is generally called an apoenzyme, and the apoenzyme plus the cofactor (i.e., the active enzyme) is called a holoenzyme.

-Enzymes possess remarkable specificity, as they generally catalyze the conversion of only one type (or at most a range of similar types) of substrate into product.

-An enzymatic reaction is often described as occurring in three steps:

$$E + S \longrightarrow \text{ES complex} \longrightarrow E + P$$

The ES complex represents a configuration where the substrate (S) binds to the enzyme (E), thus promoting the reaction. Once the reaction is complete, the product molecule (P) dissociates from the enzyme, which is then free to bind to another substrate molecule (Robinson, 2015).

- Enzymes exhibit a hyperbolic response to substrate concentration, according to the Michaelis-Menten equation :

$$v = \frac{V_{\max} \cdot [S]}{K_m + [S]}$$

Where: [S] is the substrate concentration, v is the measured speed, Vmax is the maximum speed at infinite substrate concentration and Km is the Michaelis constant.

- Enzymes are only active within a limited pH range. This range can be wide (e.g., from pH 5 to 10) or narrow (e.g., less than one pH unit). Within this range, there is an optimum at which maximum activity (the highest value of Vmax) is reached; this optimum may itself be a narrow range.

- Temperature influences enzyme activity in a similar way to other chemical reactions. Reaction rates increase by 4 to 8% per degree Celsius, although at high temperatures, enzyme denaturation reduces product formation.

- Each enzyme reacts specifically to ionic strength (salt concentration). Some enzymes are fully active at minimal ionic strength, while others require high salt concentrations for significant activity. Most are inhibited at high salt concentrations (40.5 mol/L) (Scopes, 2002).

1.2. Methods of enzyme assays

1.2.1. Spectrophotometric assays

These assays measure the changes in absorbance resulting from the formation of reaction products or the consumption of substrates. They are versatile and widely used due to their simplicity and compatibility with various enzymes and substrates (Kei, 2024).

UV-visible spectroscopy is a powerful method for studying enzymatic kinetics and interactions. By measuring the absorbance of UV or visible light, it allows us to track changes in substrate and product concentration, thus providing a real-time view of enzymatic activity. Many enzymes and substrates absorb light according to specific profiles in this spectral range, which is useful for studying their reactions. Using a spectrophotometer, we can track these changes by applying Beer-Lambert's law (see Chapter 3). When the enzyme reacts with the substrate, the amount of product increases or the amount of substrate decreases (Shukla et al., 2025).

1.2.2. Fluorometric assays

Fluorescence-based assays exploit the intrinsic fluorescence properties of certain molecules, such as NADH and some artificial substrate analogs, to track enzymatic reactions. They offer high sensitivity and specificity, making them ideal for studying enzyme kinetics and screening inhibitors (Bisswanger, 2014; Kei, 2024).

1.2.3. Electrochemical assays

Electrochemical methods consist of measuring the changes in electrical properties resulting from enzymatic reactions (Kei, 2024), particularly the determination of pH for reactions involving pH changes, such as the release of acids by lipase or cholinesterase. Since pH changes strongly influence enzymatic activity, a pH meter connected to an automatic burette is used. This device maintains a constant pH by adding a neutralizing solution, the quantity of which is directly proportional to the enzymatic activity (Bisswanger, 2014).

Continuous methods allow for real-time monitoring of the progress of an enzymatic reaction, which is essential for measuring the rate and detecting anomalies, focusing on the linear phase of the reaction. The reaction then slows down due to substrate depletion and eventually stops. When no direct signal is available, discontinuous methods are used, where the reaction is stopped at a specific time to measure the product or substrate. This approach provides only a single measurement point, requiring multiple trials to ensure that the rate is calculated within the linear phase (Bisswanger, 2014).

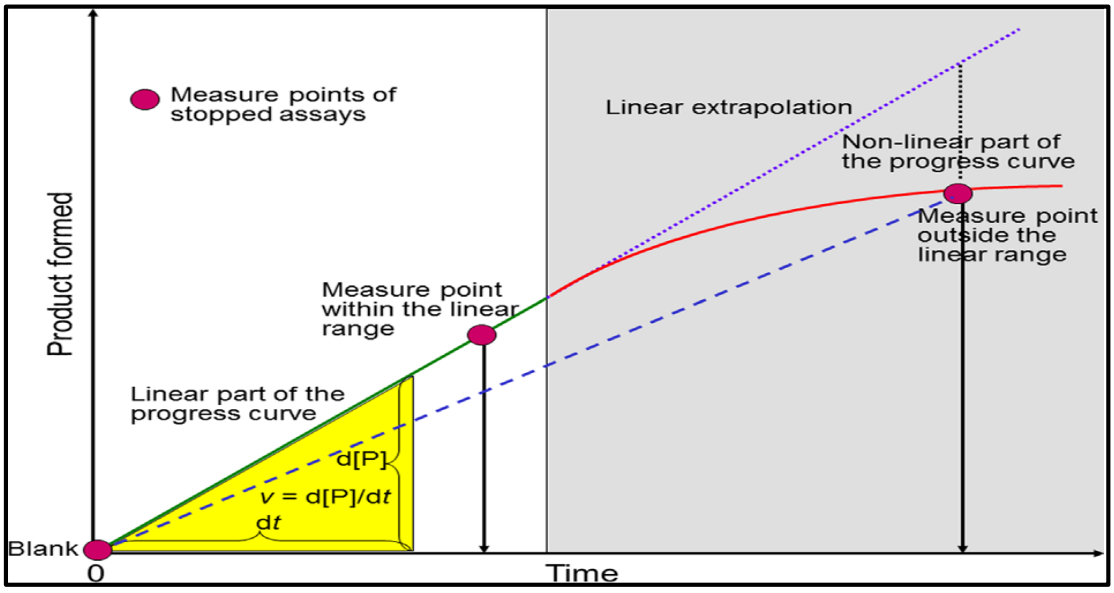

Figure 57: Progress curve of a typical enzymatic reaction. The rate is obtained from the slope of the linear portion of the curve, relative to a distinct unit of time (1 min or 1 s). Stopped trials provide only a single measurement point (Bisswanger, 2014).

1.3. Determination of enzyme activity

1.3.1. Direct assays

The reaction rate is measured continuously. Only a few analytical methods are suitable for this direct measurement in the reaction medium (e.g., visible and ultraviolet absorption spectrophotometry, spectrofluometry, etc.). In this approach, one of the products of the

enzymatic reaction absorbs light; for example, the formation of NADH by the reduction of NAD+ (NADH absorbs light at 340 nm and fluoresces at 450 nm).

1.3.2. Indirect (coupled) assay

One of the simplest methods for making an enzymatic reaction detectable involves converting the reaction product with a second enzyme to form a second product, and so on, until one of these successive reactions produces a detectable signal. The vast majority of enzyme assays involve an oxidoreductase, using NAD or NADP as cofactors (Reymond et al., 2009).

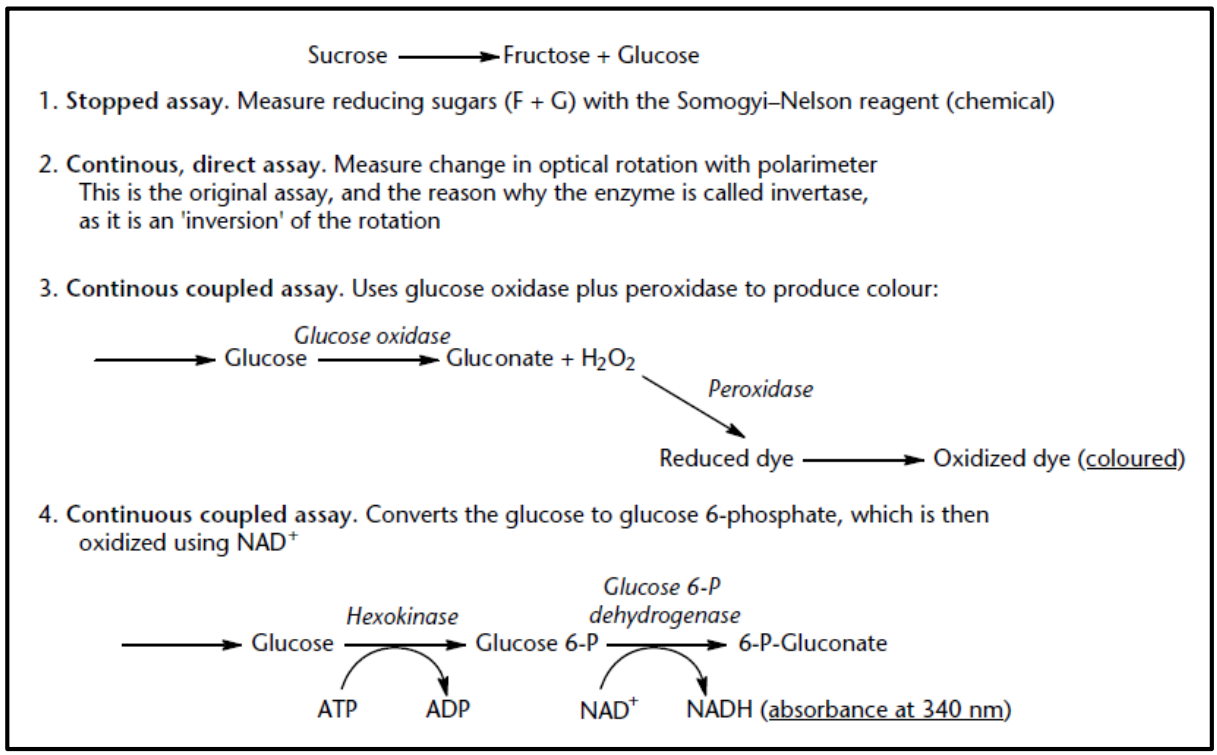

Figure 58: Four different methods for measuring the invertase enzyme (Scopes, 2002).

2. Immunological tools

Immunological methods are analytical techniques used to detect and/or quantify antigen-antibody interactions. For almost all immunological techniques, the binding of a signaling molecule to the antibody and/or antigen is essential. Numerous signaling molecules are used in immunological techniques, including iodine, enzymes, fluorochromes, and radioisotopes. These signaling molecules produce a visual signal that allows for the quantification of the specific antibody-antigen interaction being studied (Yu et al., 2015).

2. 1. Precipitation methods

2.1.1. Definition

Immunoprecipitation, commonly known as IP, involves the binding of a protein antigen in solution to an antigen-specific antibody, followed by purification of the antigen-antibody

complex by fixation onto a solid-phase matrix such as protein A or G agarose (DeCaprio and Kohl, 2020). When sufficient amounts of antibody and antigen are present, a visible precipitate forms, consisting of antigen aggregates cross-linked by antibodies. The formation of this precipitate depends on the antigen-to-antibody ratio and was used for the first quantitative antibody assay (Janeway et al., 2021).

The precipitation reaction is influenced by the number of binding sites each antibody possesses for the antigen, and by the maximum number of antibodies that can bind simultaneously to a single antigen molecule or particle. These quantities are defined as the antibody valence and the antigen valence: the antibody and antigen valences must be greater than or equal to two for precipitation to occur. The valence of an antibody depends on its structural class. An antigen will only precipitate if it possesses multiple antibody binding sites. This condition is generally met for macromolecular antigens, which have a complex surface with binding sites for several different antibodies. The site on an antigen to which each specific antibody molecule binds is called the antigenic determinant or epitope. However, steric considerations limit the number of specific antibody molecules that can bind simultaneously to a single antigen molecule, as antibody molecules binding to partially overlapping epitopes compete for space. This is why the valence of an antigen is almost always less than the number of epitopes it presents (Janeway et al., 2021).

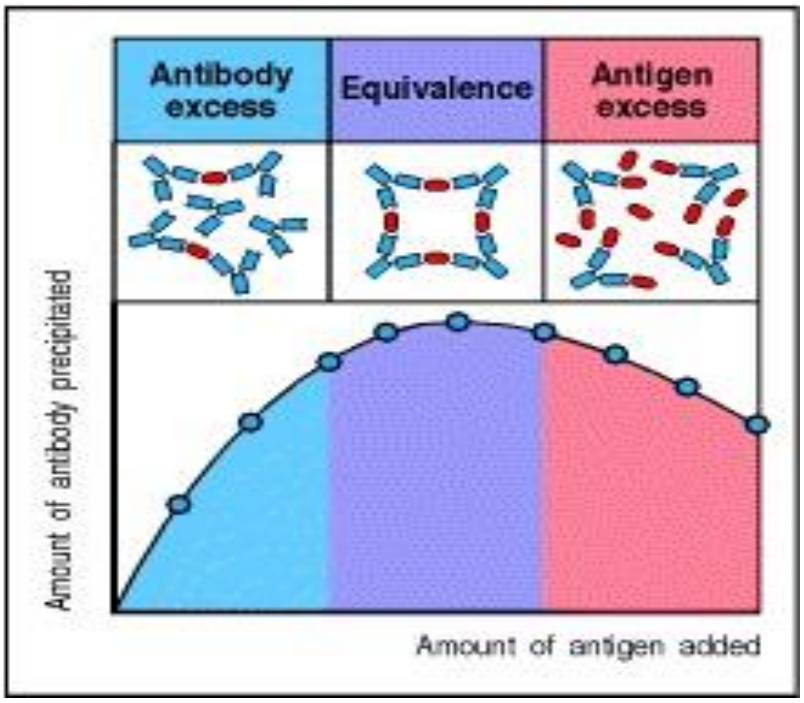

Figure 59: Precipitation curve (Janeway et al., 2021).

2. 2. Agglutination methods

2.2.1. Definition

Agglutination occurs when an insoluble or particulate antigen interacts with an antibody. A positive reaction is detectable macroscopically within a short time. However, the antigen-antibody complex may be visible to the naked eye if it is large. IgG or IgM antibodies can be involved in the agglutination reaction (Sadeghalvad and Rezaei, 2022). Agglutination reactions can be described as direct or passive tests. Direct tests involve the interaction of an antibody with a cellular antigen. Bacterial agglutination, used for typing bacterial strains and diagnosing infections, and direct hemagglutination, for determining blood groups, are examples of direct tests. Passive hemagglutination, or agglutination of coated latex particles, and the measurement of C-reactive protein in blood and tissues are examples of passive tests (Katz, 2011).

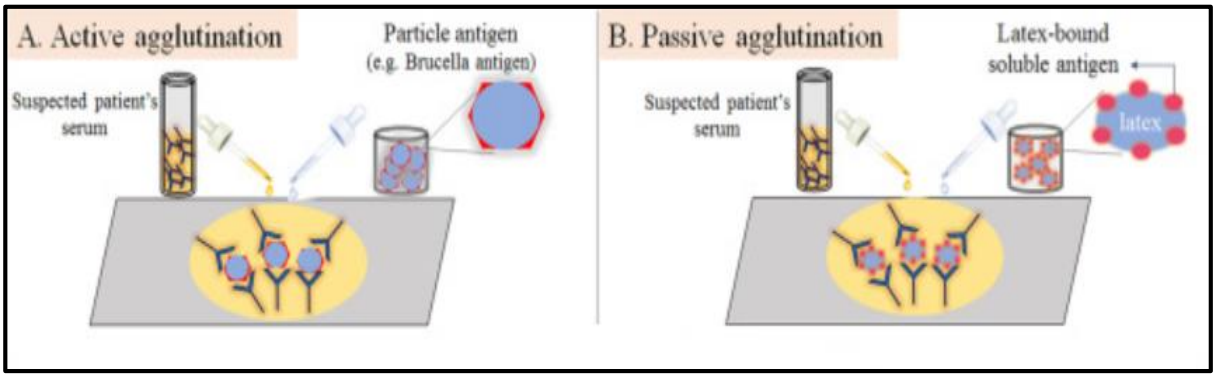

Figure 60: Types of agglutination (Sadeghalvad and Rezaei, 2022).

2. 3. Immunoelectrophoresis

2.3.1. Definition and principle

Immunoelectrophoresis is a combination of protein electrophoresis and immunoprecipitation (Levinson, 2009). Proteins are first separated by electrophoresis on a semi-solid medium (gels), and then specific proteins are visualized by an immunological reaction, namely the formation of antigen-antibody precipitin. Double immunodiffusion occurs after a prepared antiserum against the target proteins is applied to a channel cut in the gel parallel to the direction of electrophoretic separation, and distinct elliptical precipitation arcs develop with the antisera for each protein antigen (Csako, 2012).

2.4. Marker-based techniques

2.4.1. ELISA

2.4.1.1. Definition

Enzyme-linked immunosorbent assay (ELISA) is a technique used to quantify the levels of peptides, proteins, antibodies, antigens, hormones, or other biomolecules in a biological sample. It is typically performed in 96-well plates (figure 61A), allowing for the measurement of

multiple samples in a single experiment. In the ELISA technique, the antigen is immobilized on a solid surface and then bound to antibodies to form an antigen-antibody complex, where the antigen-antibody complex is bound to the enzyme. The detection signal, in the form of a color change, is formed due to the reaction between the enzyme and the substrate (figure 61B) (Swanson et al., 2019; Hidayat and Wulandari, 2020; Deepak, 2020).

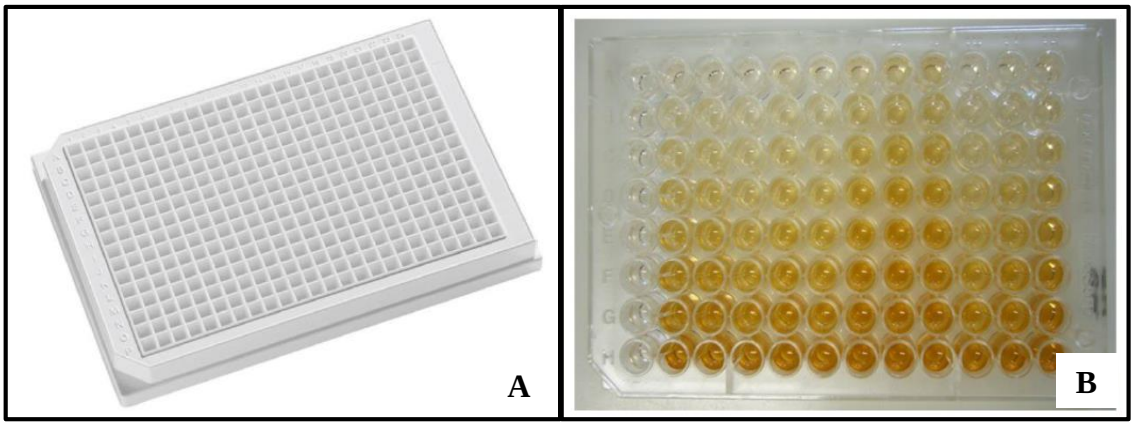

Figure 61: ELISA plate: A ; color intensity changes on ELISA plate: B (Hidayat and Wulandari, 2020).

2.4.1.2. Principle

This is an assay in which an antibody or antigen is coupled to an enzyme to specifically detect a target protein. This method combines the immunological specificity of antibodies with the sensitivity of enzymes. It allows for the detection of an analyte in a liquid sample, either using a liquid reagent or via dry strips that can be read by reflectometry. Quantification is generally performed by spectrophotometry, measuring the light intensity at a given wavelength. The sensitivity of the test depends on signal amplification, which is improved, in particular, by using high-affinity antibodies to coat the plates. There are two main variants of the ELISA method:

- An ELISA variant that can be used to detect the presence of antigens recognized by an antibody.
- Another ELISA variant that can be used to test for antibodies that recognize an antigen (Deepak, 2020).

2.4.1.3. Types

*** Direct ELISA**

Direct ELISA is used to detect protein antigens and may require pre-purification of the sample. The antigen is first fixed directly to the bottom of the wells, and then a primary antibody labeled with an enzyme is added for detection. This method is rapid and does not require a secondary antibody (Hidayat & Wulandari, 2020; Deepak, 2020).

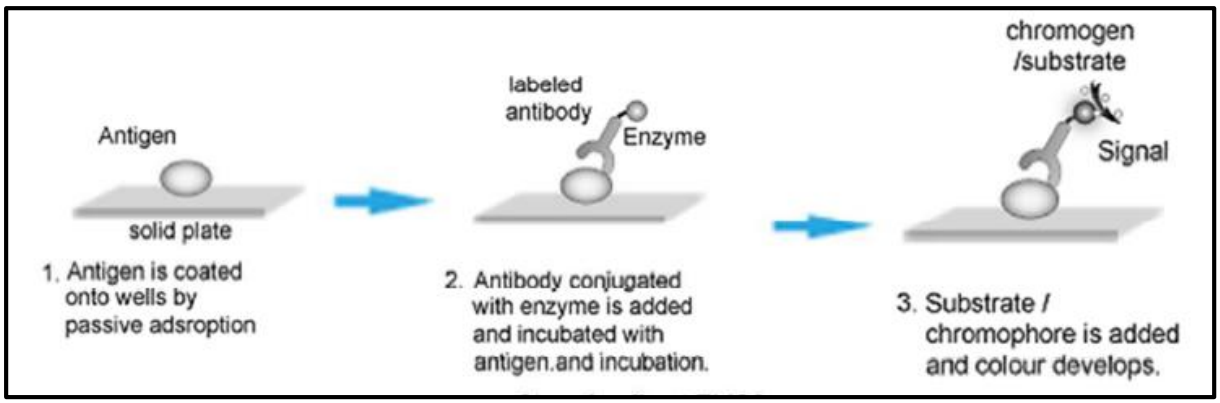

Figure 62: Steps of direct ELISA (Deepak, 2020).

*** Indirect ELISA**

An indirect ELISA is a test in which the specific antibody of the primary antigen is recognized by a secondary conjugated antibody (Swanson et al., 2019). The antigen is first passively fixed to the wells and then incubated with a specific antibody. After washing, a secondary antibody coupled to an enzyme is added to detect the bound antibody.

After a final incubation and washing, the enzymatic reaction is detected and measured by spectrophotometry (Deepak, 2020).

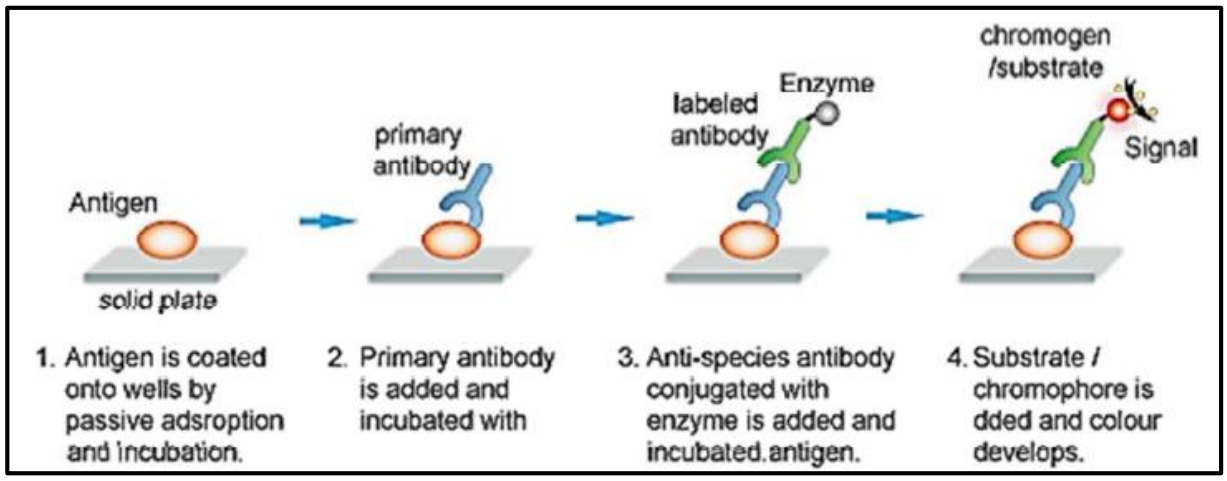

Figure 63: Steps of indirect ELISA (Deepak, 2020).

*** Sandwich ELISA**

In sandwich ELISA, the antigen is detected between two antibodies: a capture antibody bound to the well and a detection antibody bound to an enzyme. The sample is added to form an antigen-antibody complex, and then a second enzymatic antibody recognizing a different epitope binds. After washing, the addition of the substrate allows the formation of a colored product indicating the presence of the antigen. Two variants exist: direct and indirect sandwich ELISA (Deepak, 2020).

-Direct sandwich: In this method, the capture antibody is fixed to the solid phase. After removing excess unbound antibody, the antigen is added and specifically captured. The antigen is then detected by a second enzyme-labeled antibody.

- Indirect sandwich: In this method, the antigen is detected with a second unlabeled antibody. This antibody is in its turn detected using an antibody labeled with an enzyme (Deepak, 2020).

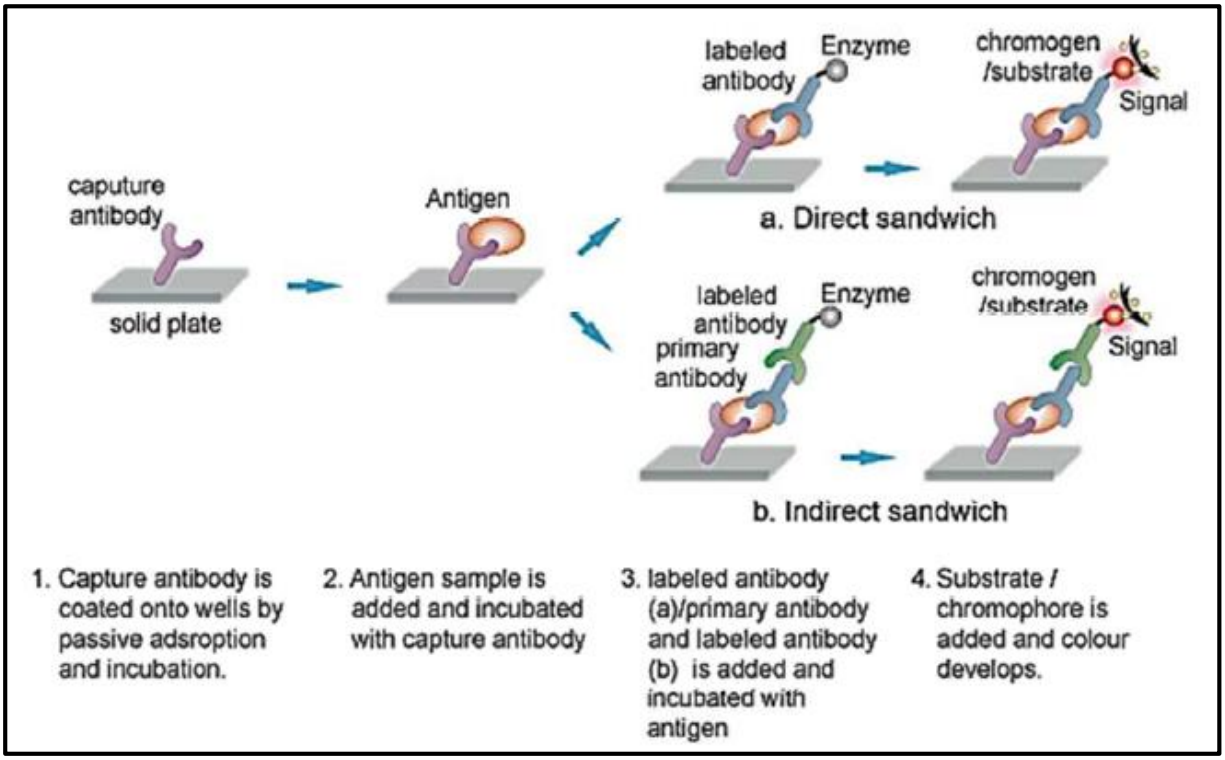

Figure 64: Sandwich ELISA steps (Deepak, 2020).

*** Competitive ELISA**

Competitive ELISA is an ELISA assay method in which there is a competitive reaction between the sample antigen and the antigen-binding agent attached to the bottom of the plate wells along with the primary antibody. In this method, the unsampled antigen is attached to the bottom of the plate. Then, the sample antigen and the primary antibody are inserted into the wells. Finally, the secondary antibodies, which are bound to the enzyme, are placed in the plate wells (Hidayat and Wulandari, 2020).

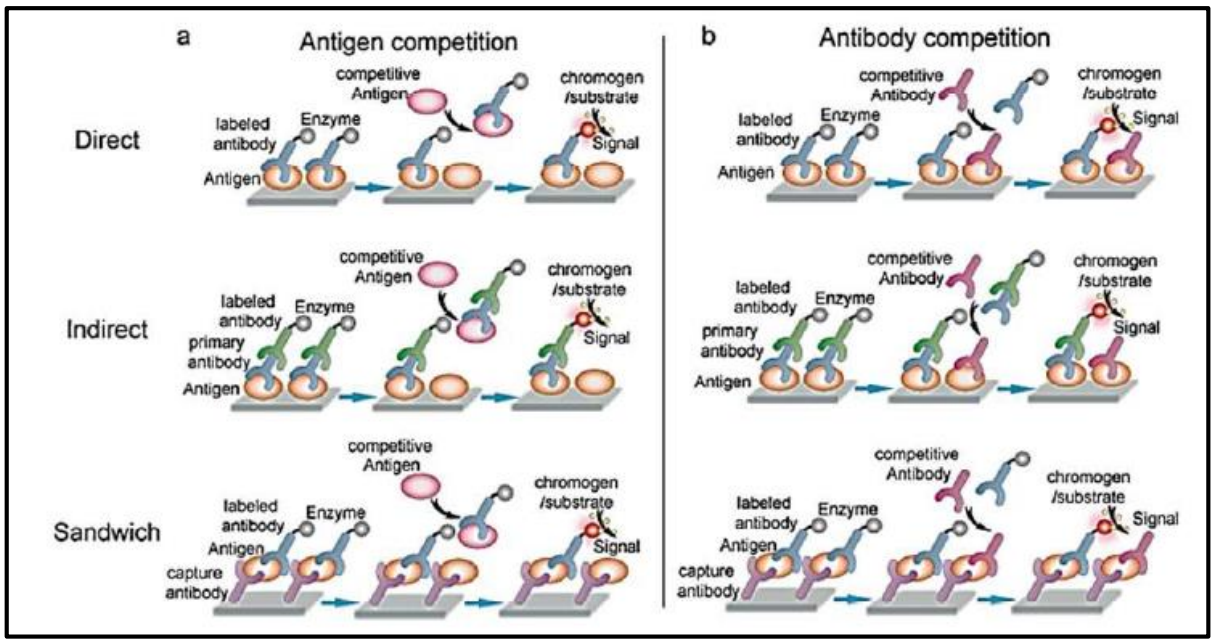

Figure 65: Steps of competitive ELISA (Deepak, 2020).

2.4.2. Western blot

2.4.2.1. Definition

Western blot, also called immunoblot, is one of the most commonly used techniques in molecular biology and proteomics in scientific laboratories. It is an analytical method for detecting and semi-quantifying target proteins. Western blot also allows the identification of specific amino acids in post-translationally modified proteins, which are altered in the cell by physiological changes, in both healthy and diseased individuals. These post-translational modifications (PTMs) of proteins can take the form of phosphorylation, ubiquitination, biotinylation, glycosylation, methylation, acetylation, sumoylation, nitration, oxidation/reduction, nitrosylation, and other types (Sule et al., 2023).

2.2.2. Principle

Western blotting (WB) allows for the quantification of protein expression and modifications in homogenized tissues or lysed cells. It relies on the use of specific antibodies targeting the protein of interest or its chemical modifications, such as glycosylation or phosphorylation. Denatured proteins are separated by gel electrophoresis, transferred to a membrane, and then detected using a specific antibody. The antigen-antibody complex is then visualized by chemiluminescence, using radiographic film or a gel-based imaging system (Vayanaperumal, 2023).

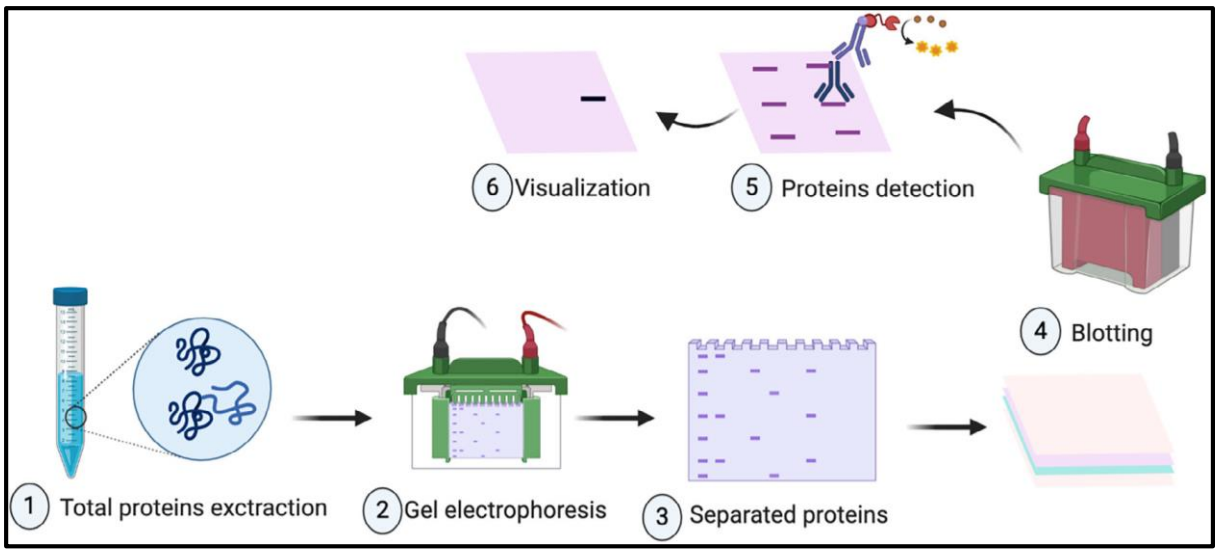

Figure 66: Principle of Western blotting (Meftahi et al., 2020).

2.4.3. Radioimmunology

2.4.3.1. Definition

Radioimmunoassay (RIA) is a highly sensitive and specific laboratory technique used to quantify the concentration of specific substances, such as hormones, drugs, and antigens, in biological samples. This innovative method has revolutionized the fields of immunology and clinical diagnostics, enabling researchers to obtain valuable information on various physiological and pathological processes (Zoey, 2023).

2.4.3.2. Principle

Radioimmunoassay (RIA) uses monoclonal or polyclonal antibodies to bind specifically to a target antigen labeled with a radioisotope (e.g., iodine-125). The sample is added, forming antigen-antibody complexes that precipitate. After separation of the complexes from unbound antigens, radioactivity is measured by gamma counter or liquid scintillation. The intensity of the radioactive signal is proportional to the antigen concentration, allowing sensitive quantification even at low concentrations (Zoey, 2023).

2.4.4. Immunofluorescence

2.4.4.1. Description

Immunofluorescence is a powerful technique that uses fluorescently labeled antibodies to detect specific target antigens. It includes direct immunofluorescence and indirect immunofluorescence. Direct immunofluorescence uses fluorescently labeled antibodies to bind directly to the target antigen. Indirect immunofluorescence uses a two-step technique: an unlabeled primary antibody binds to the target, and then a second antibody labeled with a fluorophore is used to detect the first. This technique is more complex and time-consuming than

direct immunofluorescence; however, it is more sensitive because several secondary antibodies can bind to each primary antibody, amplifying the fluorescence signal (Odell and Cook, 2013).

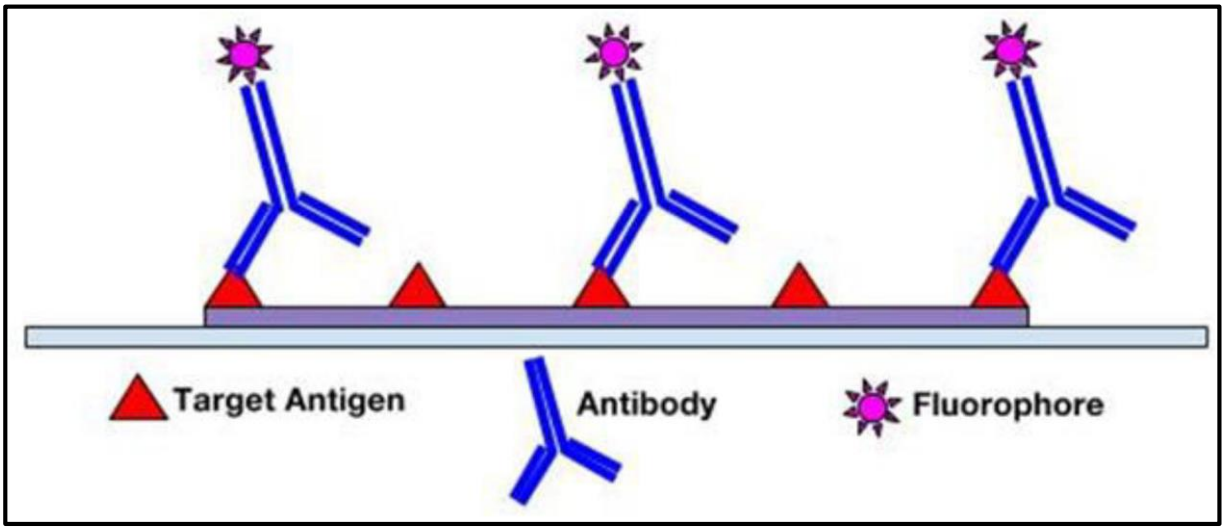

Figure 67: Direct immunofluorescence (Im et al., 2019).

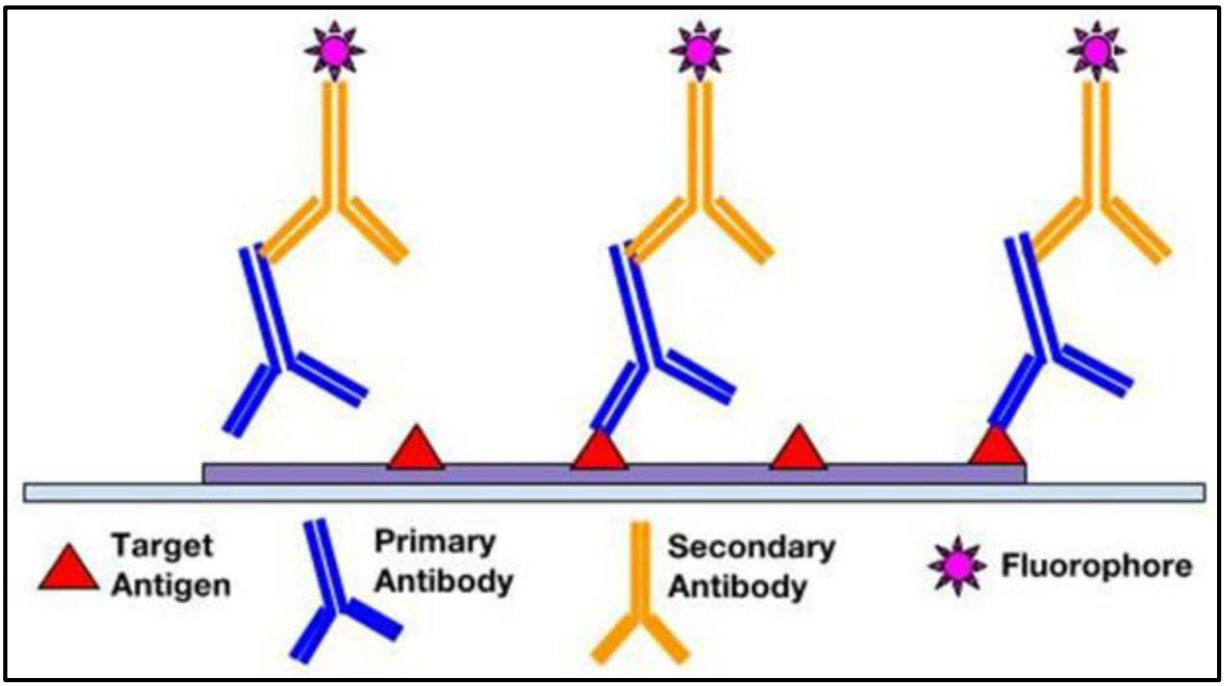

Figure 68: Indirect immunofluorescence (Im et al., 2019).

2.4.5. Immunohistochemistry

2.4.5.1. Definition

Immunohistochemistry (IHC) is an auxiliary testing method widely used in anatomical surgical pathology for the classification and diagnosis of cells and uses antibodies targeting certain antigens in specific tissues and cells to facilitate the determination of cell type and organ of origin (Magaki et al., 2019).

2.4.5.2. Principle

Immunohistochemistry (IHC) uses specific antibodies to detect antigens in cells, visualized by a chromogen or a conjugated enzyme. It can be performed using direct or indirect methods, the latter being more sensitive and popular, employing unlabeled primary antibodies and labeled secondary antibodies. The avidin-biotin complex and horseradish peroxidase enzyme with the chromogen DAB are commonly used, enabling the detection and development of new diagnostic and prognostic markers (Panicker & Mustafa, 2021).

3. Examples of application in pathophysiology

3.1. Enzymatic tools

***Phenylketonuria**

Phenylketonuria (PKU) is an inherited metabolic disorder caused by a decrease or deficiency in the activity of the liver enzyme complex phenylalanine hydroxylase. Impaired activity of this enzyme, which catalyzes the conversion of phenylalanine (Phe) to tyrosine, leads to an accumulation of Phe in the blood, which is used as a biomarker for PKU screening (Moreira et al., 2018).

For monitoring phenylalanine levels, an electrochemical enzymatic platform on paper, coupled to a screen-printed graphene-modified electrode, was developed. The biosensor's performance was measured by differential pulse amperometry. The linearity range of the enzymatic biosensor was established between 5 and 600 μM, and the limit of detection (LOD) was set at 0.2 μM. Selectivity testing was performed with bilirubin, hemoglobin, cysteine, tryptophan, tyrosine, leucine, methionine, and ascorbic acid. Only ascorbic acid induced an increase of approximately 10.2% in the background signal. However, the concentration of ascorbic acid added during the selectivity test was too high compared to physiological levels. Therefore, the method has proven to be highly sensitive to phenylalanine (Moreira et al., 2018).

***Fabry Disease**

Fabry disease is a hereditary metabolic disorder characterized by a deficiency in α-galactosidase A, a lysosomal enzyme essential for glycosphingolipid metabolism. The most commonly used enzymatic analysis method relies on the cleavage of 4-methylumbelliferyl-α-D-galactoside, a synthetic fluorogenic substrate. Recently, a substrate suitable for measuring enzyme activity by electrospray ionization tandem mass spectrometry (ESI/TMS) has also been developed. The activity of α-galactosidase A should always be determined in the presence of α-N-acetylgalactosamine, which inhibits α-galactosidase B. In addition, another lysosomal enzyme,

preferably β-galactosidase, should always be measured to assess the quality of the sample (Gal et al., 2011).

3.2. Immunological tools

*** Diabetes**

Sandwich ELISA is widely used to measure insulin, employing a capture antibody and a peroxidase-labeled detection antibody. Insulin from the sample binds to the immobilized antibodies, and the colored signal generated by the TMB substrate is measured at 450 nm to quantify the analyte. This method is selective, but some kits exhibit cross-reactivity with proinsulin, which has spurred the development of more specific assays, such as AlphaLISA and HTRF for insulin (Shen et al., 2019).

***Hypothyroidism and hyperthyroidism**

Second-generation radioimmunoassay methods, such as IRMA, allow for more sensitive TSH measurement by using two monoclonal antibodies: one immobilized and one labeled with iodine-125, forming a "sandwich" complex that is measured by radioactivity. Other methods, such as CPB and RRA, are now rarely used. Enzyme-linked immunosorbent assays (EIA, ELISA) and immunofluorescence assays (FIA) also offer high sensitivity, with a limit of detection as low as 0.01 μIU/ml for TSH (Matyjaszek-Matuszek et al., 2013).

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