Ss. CYRIL AND METHODIUS UNIVERSITY IN SKOPJE
FACULTY OF DENTISTRY - SKOPJE

ORAL BIOCHEMISTRY AND PHYSIOLOGY

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PREFACE

Almost a decade has transpired since the release of the Oral Biochemistry textbook. Over this span of time, the textbook effectively addressed the educational requirements of students, equipping them with a substantial understanding of the biochemical processes occurring within the oral cavity. Nevertheless, a decade signifies a considerable duration during which scientific thought within the realm of dentistry is unlikely to remain static. Notably, there have been significant advancements in scientific discoveries concerning the biochemical and physiological processes of oral tissues, as well as the fundamental composition of the oral milieu, particularly saliva, in the past ten years. These developments encompass the identification of novel categories of salivary peptides, the elucidation of the functions of these peptides, and their pivotal role in the maintenance of oral health. Furthermore, considerable progress has been made in the domain of scientific exploration involving saliva as a diagnostic medium.

In the upcoming edition of the Oral Biochemistry and Physiology textbook, which will be utilized by students starting from the next academic year, the latest developments in the field of dentistry have been incorporated. To compile this textbook, our own findings, obtained through an extensive series of scientific investigations, played a significant role, particularly in the examination of various constituent elements of saliva. Over the past five years, subsequent to the establishment of a well-equipped biochemical laboratory as part of the research center at the Faculty of Dentistry, we initiated an array of biochemical research projects. Among these endeavors, some were dedicated to establishing reference values for individual components found in saliva, including various electrolytes, proteins, enzymes, and degradation products. Furthermore, to enhance our comprehension of the most prevalent oral ailments, we conducted studies correlating salivary components with conditions such as dental caries and periodontal diseases.

Of particular significance are the investigations dedicated to establishing correlations between salivary components and their counterparts in the bloodstream. The primary objective of these recent research endeavors is to ascertain the viability of employing saliva as a diagnostic medium.

The publication of this textbook is the culmination of extensive, long-term studies on the intricacies of biochemical and physiological processes within the oral cavity, complemented by a multitude of laboratory investigations. These efforts provided the essential groundwork for the creation of this comprehensive resource.

The content is organized into twelve interconnected chapters, facilitating a progressive and coherent understanding of the subject matter for students. This textbook serves not only the needs of undergraduate dental medicine students but also proves valuable to those pursuing advanced studies in dentistry (second and third cycles) and trainees from various dental disciplines.

Through a comprehensive study of the educational content presented in this textbook, students will attain proficiency in the fundamental principles underlying oral biochemical and physiological processes. This proficiency, in turn, will significantly enhance their comprehension of the intricate interplay between oral biofilms and the protective functions of oral tissues and saliva. Simultaneously, the knowledge imparted by this textbook serves as the foundation for grasping the multifaceted etiopathogenic mechanisms involved in oral health issues, including periodontal disease, dental caries, as well as bacterial, fungal, and viral diseases affecting the oral cavity.

Prof. Kiro Ivanovski, PhD

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ORAL BIOCHEMISTRY AND PHYSIOLOGY

1 ORAL HOMEOSTASIS, SALIVARY GLANDS AND REGULATION OF THE SECRETION OF SALIVA

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MAJOR SALIVARY GLANDS

REGULATION OF SALIVARY SECRETION

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INFLUENCE OF DIFFERENT FACTORS ON THE QUALITATIVE CHARACTERISTICS OF SALIVA

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The significance of saliva as a biological fluid in upholding both oral and systemic health in humans is unequivocal and widely acknowledged within the fields of dentistry and medicine. Its presence in the oral cavity facilitates essential functions such as moisture regulation, articulation, mastication, swallowing, and the perception of numerous sensations. These functions collectively form the basis for an individual's overall well-being. Oral homeostasis encompasses a series of mechanisms actively involved in sustaining and safeguarding the health of all oral structures. The seamless operation of the salivary glands plays a pivotal role in enabling the maintenance of oral homeostasis.

These exocrine organs are responsible for the production of saliva. Salivary glands are classified based on size, localization, and histological structure. The glandular apparatus comprises three pairs of major salivary glands: glandulae parotis, glandulae submandibulares, and glandulae sublinguales, alongside numerous small mucous glands integrated into the oral mucosa. Notably, mucous glands are absent in the mucosa of the hard palate and gingiva.

Salivary glands produce saliva, a critical factor for oral homeostasis. Saliva represents the primary fluid within the vital oral cavity. Approximately 90% of saliva secretion is derived from the major salivary glands. The absence of saliva in the oral environment serves as a predisposing factor for numerous oral diseases.

ANATOMO-HISTOLOGICAL CHARACTERISTICS OF THE SALIVARY GLANDS

In terms of histological structure, salivary glands consist of glandular acini (Figure 1.1), which serve as the primary site for the initial secretion of future saliva. Additionally, the glands feature collecting ducts, including intercalated, i.e. inserted ducts, drainage ducts known as striated ducts, and the main excretory duct.

Within the collecting and draining ducts, the function extends beyond the mere collection and removal of saliva. These ducts play a crucial role in modifying the ionic composition of the salivary secretion. This modification process contributes to the formation of the definitive secretion, known as saliva.

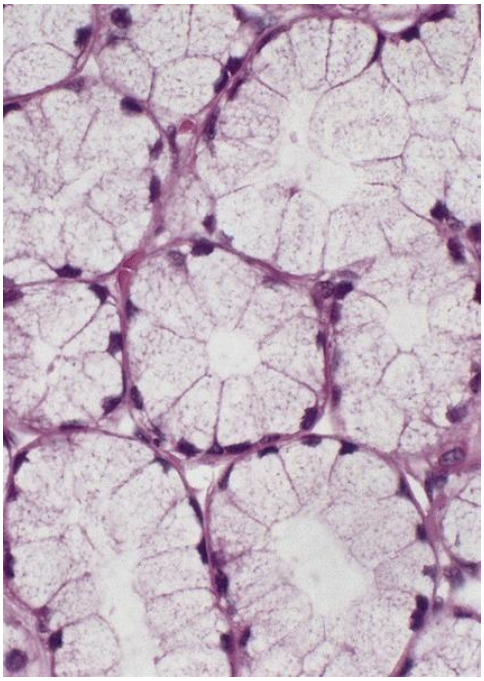


Figure 1.1: Salivary gland acini

According to the classical classification, which is based on the morphological appearance of the cells, all secretory cells within the salivary glands are traditionally categorized into two distinct groups: serous and mucous (Figure 1.2). Recent advancements in research, along with the introduction of new staining techniques and in-depth investigations into the biochemical composition of individual salivary gland secretions, have revealed limitations in this traditional classification. It has been observed that a single salivary gland often contains both mucous and serous cells, rendering the classification inadequate. Despite this recognition, the traditional classification continues to be widely accepted and can be found in the most contemporary textbooks in this field.

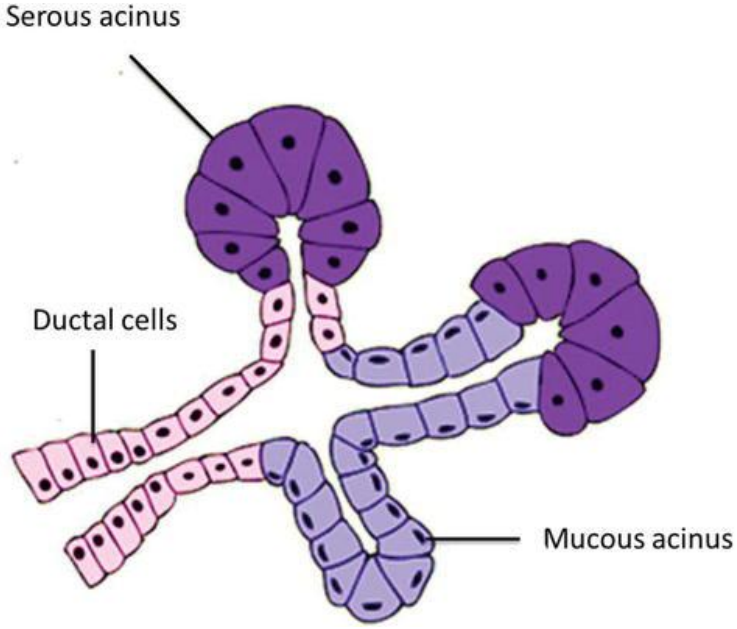


Figure 1.2: Schematic representation of serous and mucous acini

Acinar cells

Acinar cells as depicted in Figure 1.3, exhibit a pyramidal shape within the salivary glands and are organized in a circular arrangement forming a single row. This configuration results in the creation of a circular cavity known as the lumen of the acinus, illustrated in Figure 1.4. The microvillous appearance characterizes the surface of the acinar cells that form the lumen, while the basolateral surface, situated opposite, appears larger and exhibits a greater number of structural irregularities.

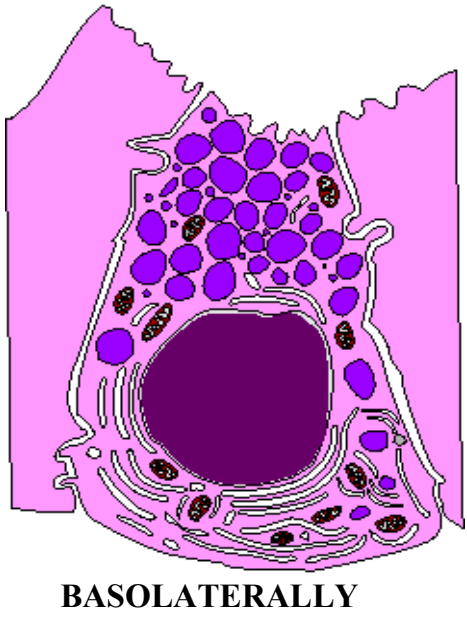


Figure 1.3: Salivary gland acinar cell: membrane, nucleus, ribosomes, mitochondria, endoplasmic reticulum and Golgi body

Intercellular channels between acinar cells are evident, constituting an extension of the acinus lumen. Desmosomal and various other cellular connections exist among acinar cells, postulated to establish a barrier between interstitial fluid and saliva within the acinus lumen.

Within the cytoplasm of acinar cells, numerous organelles (Figure 1.3), are present. The endoplasmic reticulum, forming a network of delicate membranous tubules, is situated in the basal region of the acinar cell, serving as the site for protein synthesis. The Golgi apparatus is typically observed situated above the cell nucleus, appearing as flattened sacs with vesicles at their ends. In serous acinar cells, a segment of the Golgi apparatus transforms into cisternae, where proteins undergo “packaging” before being conveyed to secretory granules. Additionally, the Golgi apparatus plays a role in the synthesis of carbohydrate chains for salivary proteins.

Within the apical part of the acinar cell, multiple secretory granules are present. The cytoplasm of acinar cells harbors a significant quantity of mitochondria, housing the cell's respiratory enzymes.

Ductal cells

The initial segments of the collecting and draining ducts are integral to the acinus structure (Figure 1.4). These segments are termed first-order ducts and are composed of cuboidal or columnar cells. These cells are characterized by a relatively low number of organelles in this ductal region.

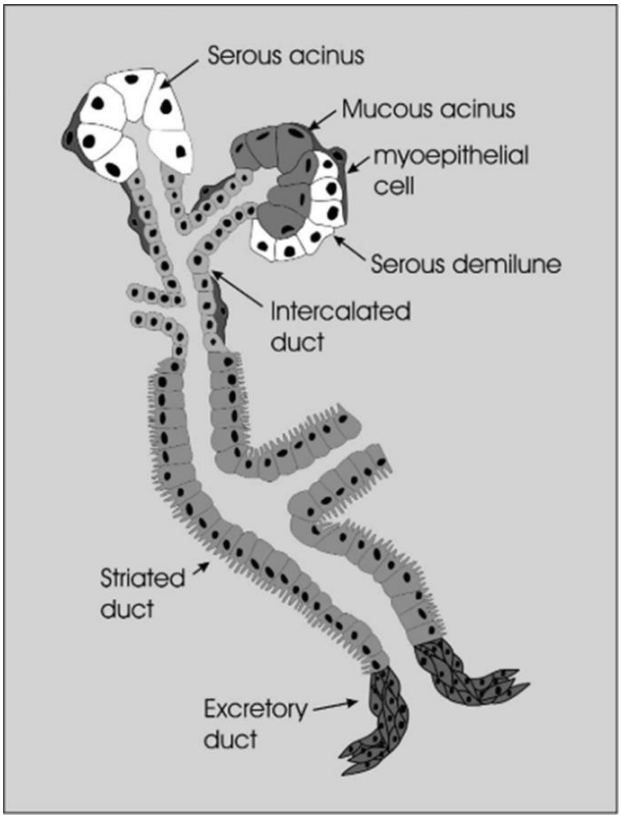


Figure 1.4. *Schematic representation of an acinar lumen with its associated collecting and excretory duct system*

The ducts, whether inserted, i.e. intercalated, primarily facilitate the flow of saliva and are not considered to play a significant role in modifying the composition of saliva.

However, in addition to their transport function, the cells of the first-order ducts actively contribute to the synthesis of specific organic components in saliva, such as lysozyme and lactoferrin. Furthermore, they play a pivotal role in the secretion of bicarbonate ions.

The subsequent segments of the collecting and draining tubules are termed second-order ducts (striated ducts). Cells within these ducts are characterized by a substantial presence of mitochondria, aligning with their distinctive function—electrolyte transport (Figure 1.5). Notably, in these channels, the reabsorption of Na+ ions from the primary saliva occurs, while K+ ions are actively excreted. This energy-demanding activity is facilitated by the mitochondria within these cells.

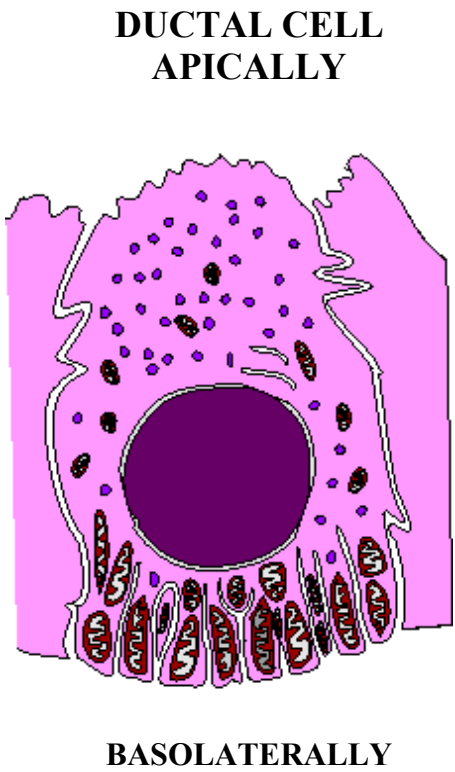


Figure 1.5: *A ductal cell containing organelles*

The main excretory duct of the salivary glands gathers saliva from the secondary ducts. Distinguishing itself from other ducts, this duct is characterized by its considerable length and the largest lumen. The main excretory duct features not only columnar cells but also basal round cells. As it progresses towards the oral opening, a stratified epithelium becomes evident in

the main excretory duct. Beyond its role in facilitating saliva flow, the main excretory duct undergoes a specific modification of the ionic composition of saliva.

Blood vessels

The salivary glands exhibit rich vascularization, as depicted in Figure 1.6. Notably, it is well-established that saliva secretion is intricately dependent on the vascular supply to the salivary gland. One or more arteries penetrate the gland, swiftly branching into arterioles. These arterioles course parallel to the glandular duct system, eventually branching into a more extensive network of capillaries. The capillary density is highest in the vicinity of the collecting ducts of the second order. The draining venous vessels follow the draining ducts of the salivary gland, and the presence of arteriovenous anastomoses is also discernible.

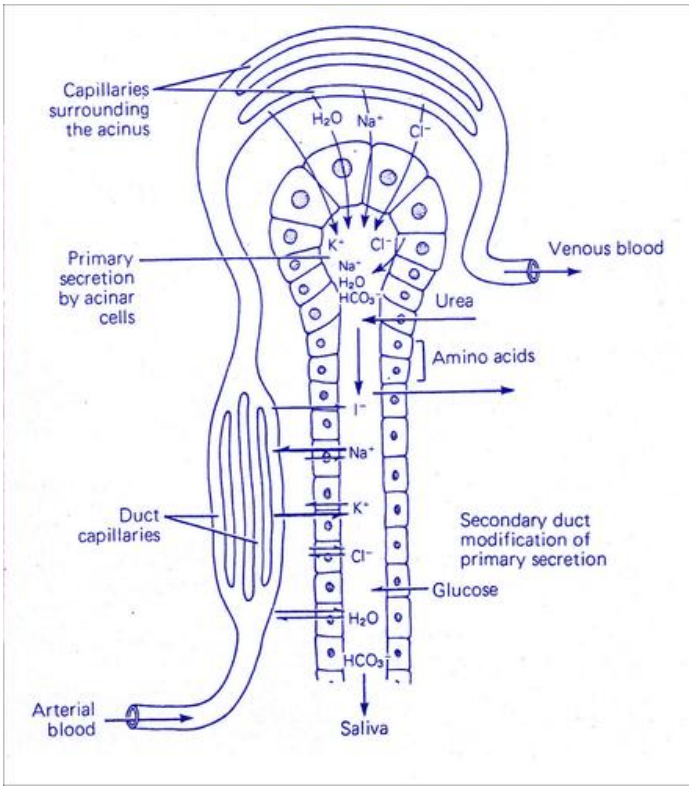


Figure 1.6: *Schematic representation of the vascular supply to the salivary glands and surrounding interstitium.*

Plasma cells are evident in the interstitium of the salivary glands. Substantial evidence supports the notion that the predominant plasma cells in this interstitial space are responsible for IgA production. It is now established with certainty that salivary IgA is locally synthesized, originating from the plasma cells within the interstitium of the salivary glands.

Furthermore, the interstitium may also feature the presence of lymphocytes, macrophages, and polymorphonuclear leukocytes. These cell types are recognized as contributors to protective mechanisms that help the human body maintain a stable internal environment.

Innervation

The innervation of the salivary glands encompasses both sympathetic and parasympathetic vegetative fibers. Parasympathetic stimulation induces vasodilation (expansion of blood vessels) within the salivary glands, leading to an increased secretion of saliva. Conversely, sympathetic stimulation produces the opposite effect, causing vasoconstriction (tightening of blood vessels) and a reduction in the volume of secreted saliva. Consequently, in stressful situations triggering sympathetic activation, saliva secretion is significantly diminished, giving rise to the uncomfortable sensation of dry mouth.

MAJOR SALIVARY GLANDS

The major salivary glands, alongside the small mucous glands, release their secretions into the oral cavity, resulting in the amalgamation of secretions from various glands. Saliva, a composite product derived from the secretions of the three pairs of major salivary glands (parotid, submandibular, and sublingual), small mucous glands, and gingival fluid, is formed through this process. This amalgamated secretion is known as mixed saliva. The physiological process of saliva secretion is termed salivation.

The three pairs of major salivary glands include (Figure 1.7):

1. Glandulae parotis (parotid glands) with an average volume of 21.6 ml (Figure 1.7):

2. Glandulae submandibularis (submandibular glands): exhibit an average volume of 6.5 ml;

3. Glandulae sublingualis (sublingual glands): these glands showcase an average volume ranging from 3 to 4 ml;

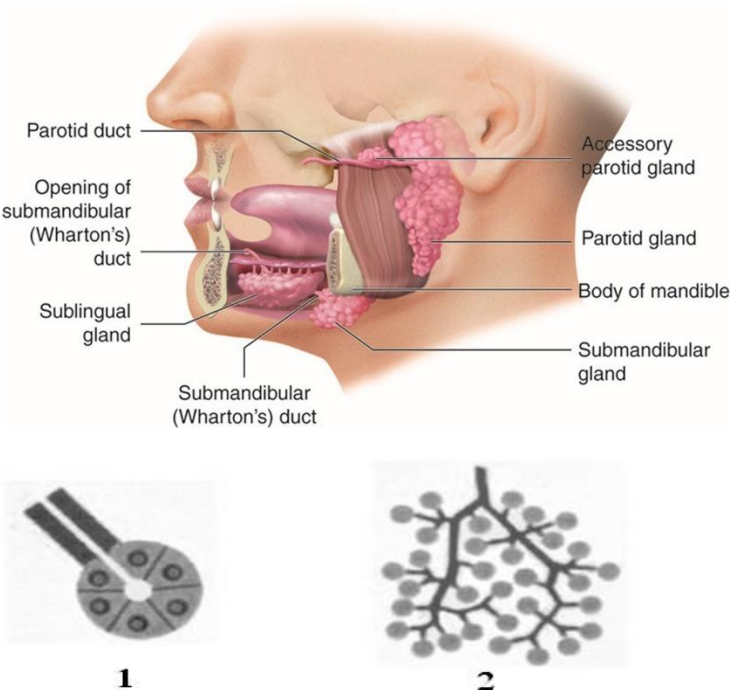


Figure 1.7. *Major Salivary Glands: Salivary glands are exocrine glands comprised of serous cells arranged around a single acinus in a minor salivary gland (1) or a cluster of acini with a shared collecting duct in a major salivary gland (2).*

The parotid glands, situated retromolarly in the fossa parotidea, stand as the largest salivary glands. Their average weight spans from 15 to 30 grams, and their dimensions measure 6 by 4 centimeters. The excretory duct for these salivary glands, known as ductus parotidei or Stensen's duct, is notably lengthy,

traversing through the masseter muscle and ultimately opening into the oral cavity along the buccal mucosa, in proximity to the first or second maxillary

molar (Figure 1.8). Predominantly producing serous saliva, the secretion is characterized by a low percentage of mucin, a high molecular weight glycoprotein. This composition imparts a low viscosity to the parotid saliva.

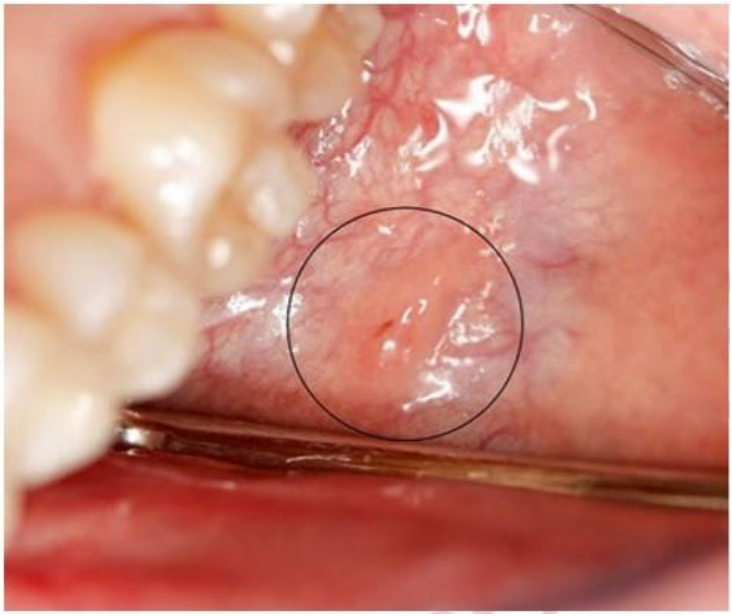


Figure 1.8: *Drainage duct of parotid salivary glands (ductus parotidei- Stennoni)*

The submandibular salivary glands, comparatively smaller than the parotid glands, possess an average weight ranging from 7 to 10 grams. Secretion from the submandibular glands is released through Wharton's duct into the oral cavity, specifically at its base, adjacent to the lingual frenulum. These glands produce seromucous saliva, characterized by higher viscosity due to an increased amount of mucin.

In both the parotid and submandibular glands, alongside secretory (acinar) cells, myoepithelial cells are also present, positioned between the secretory cells and the basal lamina (Figure 1.9).

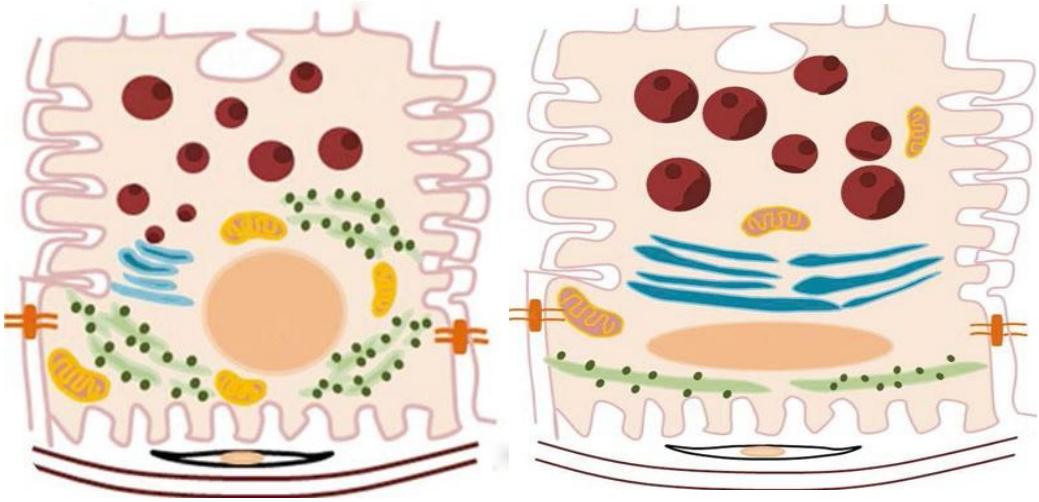


Figure 1.9: *The left side illustrates an individual serous acinar cell with its complete set of organelles. Positioned between the acinar cell and the membrane is a myoepithelial cell; on the right side, a single mucous acinar cell is depicted, featuring all its organelle; a myoepithelial cell is interposed between the acinar cell and the membrane*

Myoepithelial cells play a crucial role with their contractile function. Specifically, upon stimulation of saliva secretion, these cells induce contraction and exert pressure on the acinar cell, providing the strength necessary to propel the secretion from the acinus lumen into the canalicular system of the salivary gland. Additionally, myoepithelial cells play a significant role in the depolarization process of the acinar cell membrane, ensuring the transmission of signals for saliva secretion.

The sublingual glands are equipped with one primary draining duct, known as Bartholin's duct, and occasionally, several smaller ducts (dd. Rivini). These openings are situated on the floor of the oral cavity adjacent to the frenulum of the tongue (Figure 1.10). The secretion from these glands is notably mucous, characterized by a substantial amount of mucin.

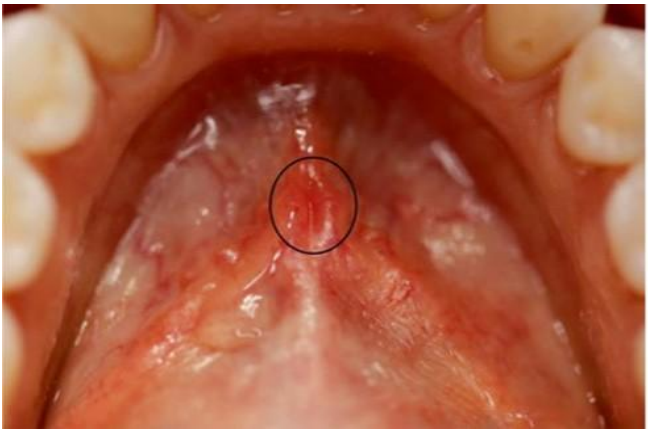


Figure 1.10: *Drainage ducts of the submandibular and sublingual salivary glands*

REGULATION OF SALIVARY SECRETION

Mixed saliva serves as a mechanism for oral homeostasis, with its secretion being under the regulatory influence of both the endocrine and central nervous systems.

From the endocrine perspective, the adrenal gland cortex, as part of the endocrine system, significantly contributes to saliva secretion by releasing various hormones into the bloodstream. Among these hormones, aldosterone holds particular importance in the process of salivation. At the level of the collecting and draining ducts of the salivary glands, aldosterone plays a role in the regulation of sodium and potassium metabolism. By influencing sodium metabolism, aldosterone indirectly contributes to the regulation of chloride metabolism. It indirectly influences chloride metabolism through its role in sodium regulation. Under the influence of aldosterone, sodium ions (Na^+) are reabsorbed into the bloodstream, while potassium ions (K^+), serving as substitutes for sodium, are secreted into the saliva. Aldosterone's impact on saliva composition results in saliva being the bodily fluid with the highest potassium concentration. The potassium content in saliva can be three times higher than that in serum, highlighting the significant role of aldosterone in modulating salivary electrolyte levels.

Catecholamines, particularly adrenaline, a hormone originating from the adrenal gland's medulla and sympathetic nerve endings, exert a notable impact on saliva secretion. Through binding to α-receptors situated on the endothelial cells of blood vessels, including those within the salivary glands, catecholamines

induce vasoconstriction, resulting in vessel narrowing. Consequently, this constriction diminishes the blood flow through the glandular parenchyma, leading to a significant reduction in the volume of secreted saliva. During periods of heightened mental stress, characterized by an elevated release of adrenaline into the bloodstream, a distinct type of saliva, characterized by its reduced volume and increased viscosity, is produced. This variant is referred to as "sympathetic saliva."

Insulin, a hormone originating from the pancreas, plays a vital role in regulating the normal metabolism of carbohydrates and fats, and it also has an indirect impact on salivation. In instances of diabetes, where insufficient insulin is secreted or inadequately released into the bloodstream, characteristic clinical manifestations of the disease emerge, including polyuria (increased urine output), polydipsia (excessive thirst), and polyphagia (uncontrollable hunger). Under such circumstances, heightened water loss and body dehydration occur, resulting in a reduction of salivary production and the onset of dryness in the oral cavity.

The central nervous system plays a pivotal role in the intricate regulation of salivation, with three distinct centers governing the function of the salivary glands:

1. Primary center of salivation, located in the medulla oblongata.

2. Secondary center of salivation, located in the thalamus, which serves as a crucial brain junction for for sensitive nerves

3. Tertiary center of salivation, positioned in the opercular-insular zone of the cerebral cortex

The primary center of salivation comprises the superior and inferior salivatory nuclei (nucleus salivatorius superior et inferior). These nuclei establish neuronal connections with the nucleus tractus solitarii where the nuclei of the seventh, ninth, and tenth cranial nerves are situated. The salivary glands are linked to the primary salivary center through the dendrites of these nerves, and the primary and secondary salivary centers are interconnected through the axons of these nerves (Figure 1.11). Nerve fibers originating from the secondary center of salivation extend to the opercular-insular zone of the cerebral cortex (fissura Sylvii zone), constituting the tertiary center of salivation.

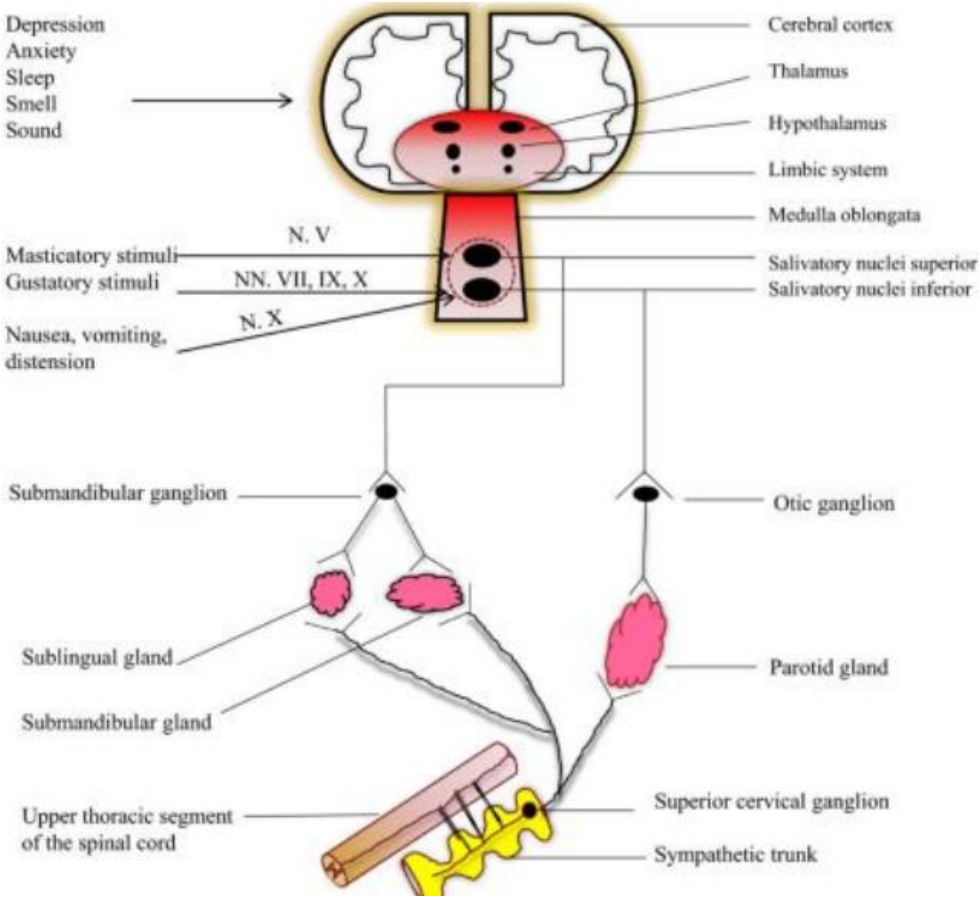


Figure 1.11*: Schematic representation illustrating the neural regulation of salivation,*

Upon stimulation of each of the mentioned centers, an increased secretion of saliva can be achieved. Numerous studies have consistently demonstrated that the primary center of salivation holds paramount importance in salivary regulation. For instance, electrical stimulation of this center results in a remarkable 30-fold increase in the volume of secreted saliva compared to the resting phase.

A notable connection exists between the salivary centers and the center responsible for the sense of taste, known as the gustatory zone. Specifically, the center of the sense of taste shares the same localization as the tertiary center of salivation.

This unequivocally underscores the regulatory role of the highest structures of the central nervous system in the intricate process of salivation.

The quantity and quality of secreted saliva are influenced by various factors, including:

- The condition within the oral cavity.
- The condition of the primary center of salivation.
- The condition of specific parts of the cerebral cortex.

As a result of these factors, saliva, specifically mixed saliva, can be broadly categorized into two types: unstimulated saliva and stimulated saliva.

Unstimulated whole saliva is a product of the entire glandular apparatus under conditions of non-stimulation, meaning when no nutritional substances impact the gustatory and other receptors in the oral cavity. However, this definition should be approached critically because truly unstimulated saliva is nearly non-existent, especially in the awake state when the cerebral cortex is fully active. It is well-established that the state of the primary salivary center in the medulla is contingent upon the activity of higher centers in the thalamus and cerebral cortex. A direct consequence of the influence of these higher centers on the primary center of salivation is characterized by increased salivation even in the absence of stimuli in the oral cavity.

The condition of the cortex of the cerebrum and other centers significantly influences the activity of the primary center of salivation, as evidenced by data obtained through the cannulation of salivary ducts to collect and measure saliva quantity. Research has established a distinct daily rhythm of salivation. According to this rhythm, saliva secretion essentially ceases from midnight to 6 in the morning, followed by a spontaneous increase until 6 pm, when unstimulated salivation reaches its peak. Subsequently, there is a period of reduced saliva secretion until midnight when the secretion of saliva comes to a halt (see figure 1.12).

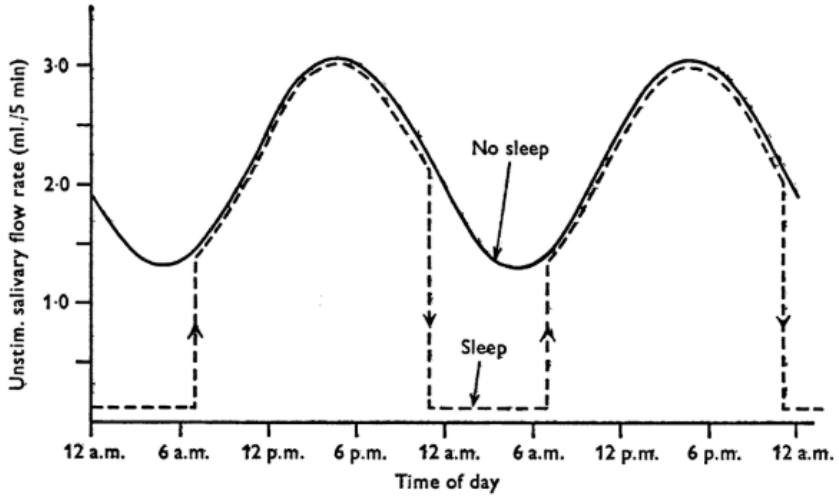


Figure 1.12: Salivation diagram of unstimulated saliva over 24 hours

The absence of practically no saliva secretion between midnight and 6 in the morning carries a dual significance:

1. This observation substantiates that the condition of the cerebral cortex profoundly influences the activity of the primary center of salivation in the medulla oblongata.

2. During this period, the self-cleaning effects in the oral environment are minimal, oral hygiene is compromised, and the potential for bacterial growth and oral health challenges is heightened.

The secretion of individual salivary glands is not directly proportional to their size or volume. Studies have revealed that, despite not being the largest glands, the submandibular salivary glands contribute the most to the daily secretion of unstimulated saliva. Specifically, 65% of unstimulated saliva is derived from the submandibular glands, 23% from the parotid glands, 4% from the sublingual glands, and 8% from the small mucous glands (see Figure 1.13). The secretion of unstimulated saliva occurs in a relatively modest amount, averaging around 0.3 ml/min.

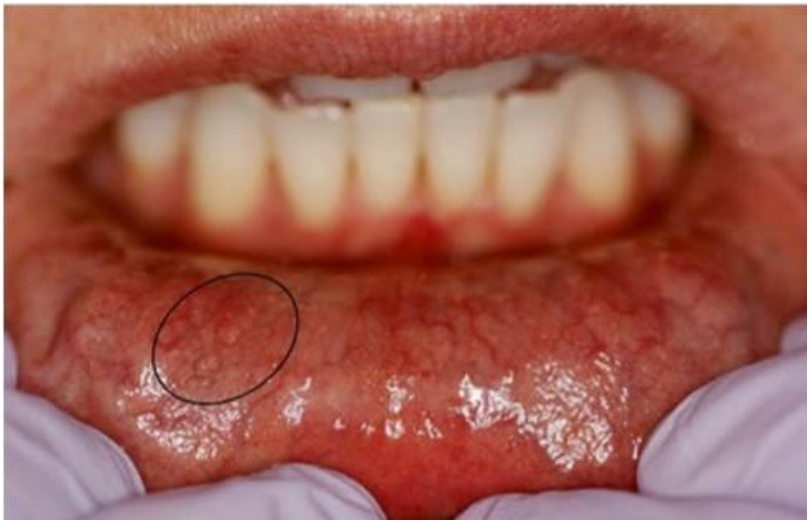


Figure 1.13: *Small mucous glands located in the labial mucosa of the lower lip*

Saliva that is produced under the stimulation of numerous receptors in the oral cavity is termed stimulated saliva. This type of secretion is significantly higher, ranging from 1.5 to 2.0 ml/min, compared to unstimulated saliva. Notably, the parotid gland, despite being the largest in volume, plays the most substantial role in the production of stimulated saliva. The parotid gland can generate a considerable volume of salivary secretion in a relatively short duration, owing to its substantial size. The contribution of the parotid glands to the production of stimulated saliva exceeds 50%.

There are several ways to stimulate the secretion of saliva, with mechanical stimulation being the simplest. Mechanical stimulation involves applying pressure to the mechanoreceptors located in the oral mucosa. When these receptors experience pressure, they send impulses along the nerve path to the salivation center, resulting in a significant increase in the salivary flow rate. Gustatory stimulation, achieved by stimulating taste receptors on the upper surface of the tongue, represents the most intense form of saliva secretion. Various acidic substances serve as potent gustatory stimuli. Additionally, psychic secretion of saliva, linked to the sense of sight and/or smell, has been observed. Merely thinking about food, seeing a delectable meal, or catching a whiff of a favorite dish can trigger an increased secretion of saliva in the oral cavity.

Research involving blind individuals, focusing on the volume of secreted saliva, has indicated that these individuals exhibit a saliva secretion volume up to 75% lower than that of healthy subjects. This underscores the significant influence of the cerebral cortex on saliva secretion. Considering the daily rhythm of saliva secretion, the total salivary flow rate of mixed whole saliva over a 24-hour period is approximately 700-800 ml.

INFLUENCE OF DIFFERENT FACTORS ON THE QUANTITY OF SALIVA

Unstimulated saliva

The volume of unstimulated saliva secretion is influenced by various factors, including mental stimulation, hydration, medications, the cicardian rhythm, body position, and exposure to light. Notably, gender, age, and body weight do not significantly affect the volume of unstimulated saliva secretion.

Mental Stimulation. The mere thought of food, as well as exposure to the sight or smell of a favorite dish, can lead to an increase in salivation. Conversely, mental stress, characterized by the secretion of catecholamines and vasoconstriction of blood vessels, can result in a reduction in saliva secretion.

The amount of water in the body. This is a critical factor significantly impacting the volume of unstimulated saliva secretion. A reduction in the body's water content by 8% can nearly eliminate the secretion of unstimulated saliva. Conversely, an increased water intake leads to an augmentation of unstimulated saliva secretion.

Medications. Many drugs can cause a decrease in saliva secretion, leading to a condition known as xerostomia. Medications associated with reduced saliva secretion include narcotics, anticonvulsant drugs, antiemetics, drugs for Parkinson's disease, antipsychotics, antidepressants, antihistamines, antihypertensives, antiarrhythmics, anxiolytics, diuretics, expectorants, sedatives, and various others.

The cicardian rhythm. As stated previously the volume of unstimulated saliva varies according to the cycardian rhythm. Saliva is secreted with the lowest intensity from midnight to six in the morning. Subsequently, the secretion gradually increases, reaching its maximum around 6 pm. In the evening, the volume of saliva secretion gradually decreases until reaching the lowest level at midnight.

Body position and exposure to light. These are also influential factors in the volume of secreted unstimulated saliva. The secretion of saliva varies significantly based on whether an individual is lying, sitting, or standing. Additionally, studies have confirmed that saliva secretion is affected by the presence or absence of light.

Stimulated saliva

This form of salivary secretion is triggered by masticatory, gustatory, or other forms of stimulation. The quantity of secreted stimulated saliva is notably higher compared to unstimulated saliva, ranging from 1.5 to 7.0 ml/min according to different authors.

Various factors influence the secretion of stimulated saliva, including the type of stimulation, size of the salivary glands, age, smoking, and vomiting.

Stimulation type. Chewing an inert substance (mechanical stimulation) that does not affect the sense of taste such as paraffin balls, induces increased saliva secretion. However, a significantly there is a higher salivary flow rate when gustatory receptors are stimulated (gustatory stimulation). The most substantial volume of salivary secretion is achieved with the use of citric acid (7 ml/min).

Size of salivary glands. The secretion of stimulated saliva is also influenced by the size of the salivary glands. As mentioned earlier, the largest salivary gland, the parotid gland, possesses the greatest capacity to produce a substantial amount of saliva in a short period. However, it's worth noting that the secretion of unstimulated saliva is not contingent on the size of the salivary glands.

Age. Regarding age, there was a historical belief that salivary secretion decreases with age, primarily based on studies conducted on hospitalized subjects who were undergoing specific medication regimens. More recent research, however, has indicated that aging has minimal impact on the secretion of both stimulated and unstimulated saliva in healthy, medication-free individuals. It is now considered that the decline in salivary secretion in older individuals is more likely associated with the intake of numerous medications rather than being a direct consequence of aging.

Vomiting. Vomiting leads to a significant increase in the salivary flow rate. However, it's important to note that the heightened buffering capacity of stimulated saliva is still insufficient to neutralize the harmful effects of hydrochloric acid on tooth enamel.

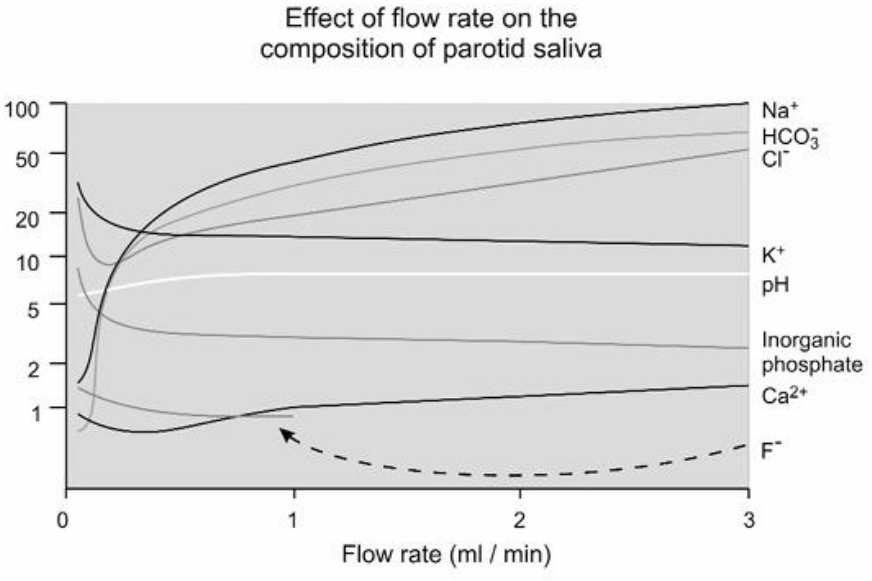
INFLUENCE OF DIFFERENT FACTORS ON THE QUALITATIVE CHARACTERISTICS OF SALIVA

Salivary flow rate

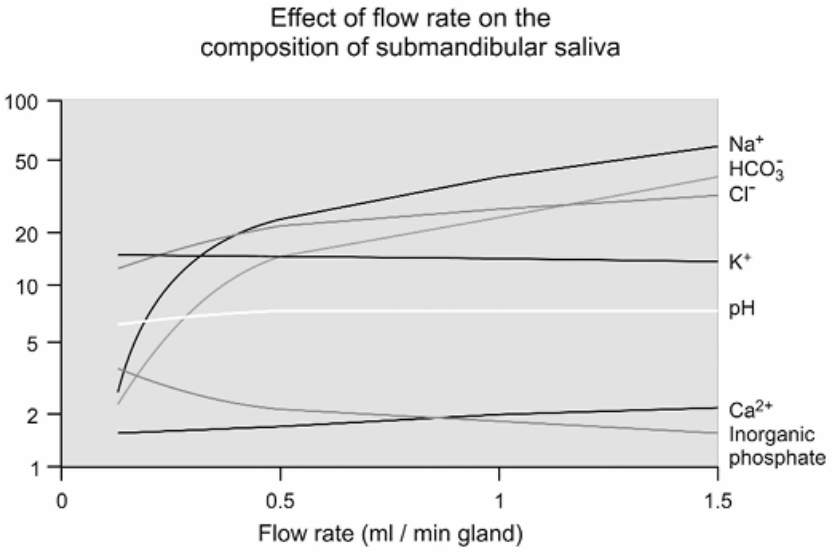
As the salivary flow rate increases, the pH-value of the saliva, as well as the concentration of certain constituent components such as proteins, sodium, chlorides, and bicarbonates, also increase. Simultaneously, there is a decrease in other constituent components, specifically magnesium and phosphates (refer to Table 1.1, Chart 1.1, and Chart 1.2).

Table 1.1: Difference in concentration of components in stimulated and unstimulated saliva

	Unstimulated	**Stimulated**
Water	99,55%	99,53%
Solid substances	0,45%	0,47%
	Average ± S.D.	**Average ± S.D.**
Salivary flow rate	0,32 ± 0,23	2,08 ± 0,84
pH	7,04 ± 0,28	7,61 ± 0,17
Inorganic components		
Sodium (mmol/L)	5,76 ± 3,43	20,67 ± 11,74
Potassium (mmol/L)	19,47 ± 2,18	13,62 ± 2,70
Calcium (mmol/L)	1,32 ± 0,24	1,47 ± 0,35
Magnesium (mmol/L)	0,20 ± 0,08	0,15 ± 0,05
Chlorides (mmol/L)	16,40 ± 2,08	18,09 ± 7,38
Bicarbonates (mmol/L)	5,47 ± 2,46	16,03 ± 5,06
Phosphates (mmol/L)	5,69 ± 1,91	2,70 ± 0,55
Thiocyanates (mmol/L)	0,70 +/- 0,42	0,34 +/- 0,20
Iodides (µmol/L)		13,8 ± 8,5
Fluorides (µmol/L)	1,37 ± 0,76	1,16 ± 0,64
Organic components		
Total proteins (mg/L)	1630 ± 720	1350 ± 290
Secretory IgA (mg/L)	76,1 ± 40.2	37,8 ± 22,5
MUC5B (MG_1) (mg/L)	830 ± 480	460 ± 200
MUC7 (MG_2) (mg/L)	440 ± 520	320 ± 330
Amylase (U/L)	317 ± 290	453 ± 390
Lysozyme (mg/L)	28,9 ± 12.6	23,2 ± 10,7
Lactoferrin (mg/L)	8,4 ± 10.3	5,5 ± 4,7
Statherin (µmol/L)	4,93 ± 0.61	
Albumin (mg/L)	51,2 ± 49.0	60,9 ± 53,0
Glucose (µmol/L)	79,4 ± 33.3	32,4 ± 27,1
Lactate (mmol/L)	0,20 ± 0.24	0,22 ± 0,17
Total lipids (mg/L)	12,1 ± 6.3	13,6
Amino acids (µmol/L)	780	567
Urea (mmol/L)	3,57 ± 1.26	2,65 ± 0,92
Ammonia (mmol/L)	6,86	2,57 ± 1,64



Graph 1.1: *The effect of the salivary flow rate on the composition of saliva excreted from the parotid gland*



Graph 1.2: *The effect of the salivary flow rate on the composition of saliva excreted from the submandibular gland*

Length of stimulation

When saliva secretion occurs smoothly and continuously, the composition of the saliva is influenced by the duration of the stimulation. Saliva secreted during a 2-minute stimulation differs in composition from saliva secreted during a 10-minute stimulation. Notably, the concentration of bicarbonate progressively increases with the duration of the stimulation. Conversely, the chloride concentration, after an initial short increase, decreases as the stimulation persists. Additionally, the composition of saliva is influenced by whether a specific gland was stimulated in the previous hour.

Nature of the stimulus

Various stimuli can influence the composition of saliva, primarily through their impact on the salivation rate. During experiments testing the influence of four different taste stimuli (sour, salty, bitter, and sweet) on parotid saliva secretion, it was determined that these stimuli do not affect the composition of electrolytes in the saliva. However, it has been established that the stimulation of secretion with a salty taste increases protein secretion in parotid saliva. Other taste stimuli are confirmed to influence the concentration and types of salivary proteins (refer to the Salivary Proteins chapter). Stimulation of receptors with a sour taste results in the largest increase of salivary flow rate and leads to the alkalinization of salivary secretion. It was previously assumed that this was an adaptive response to the nature of the stimulus, but current knowledge indicates that the pH value of saliva depends on the salivary flow rate and not on the nature of the stimulus.

2 MECHANISM OF SALIVA SECRETION, BIOCHEMICAL COMPOSITION OF SALIVA

MECHANISM OF WATER AND ELECTROLYTES SECRETION

Calcium and phosphate secretion

BIOCHEMICAL COMPOSITION OF SALIVA

Saliva and perception of taste stimuli

Calcium and phosphate concentration in saliva

MECHANISM OF WATER AND ELECTROLYTES SECRETION

The primary source of saliva are the salivary glands, including the three major pairs (parotid, submandibular, and sublingual) and numerous small mucous glands dispersed in the oral mucosa of the lips, cheeks, soft palate, and the tongue. The glands located on the tongue are referred to as Von Ebner's glands. Key structural components of the salivary glands include the glandular acini. These acini consist of specialized epithelial cells arranged to form a cavity, where the initial saliva production, involving ultrafiltration of blood plasma, takes place.

The acinar cell itself (Figure 2.1) is divided into two distinct parts: the basolateral and lumenal (apical) regions.

- The basolateral part faces the interstitium, where a network of blood vessels is situated.

- The lumenal part is directed towards the acinus lumen, where the primary saliva accumulates.

On the basolateral membrane of the acinar cell, various ion exchangers and channels are present, including Na^+/K^+ -ATPase, $Na^+/K^+/Cl^-$ cotransporters, and the Ca^{2+}-dependent K^+ channel. The most significant channel on the apical part of the acinar cell is the Ca^{2+}-dependent channel for the Cl^- ion.

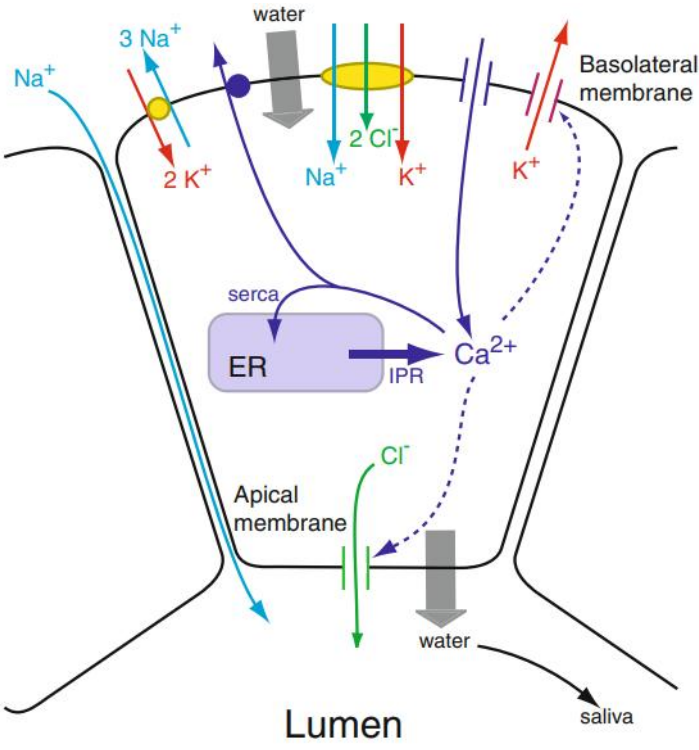


Figure 2.1: *Schematic representation of an acinar cell with its apical and basolateral parts*

The function of acinar cells is influenced by the composition of the interstitial fluid, which is directly linked to blood flow through blood vessels and capillary permeability. Primary saliva, formed by the ultrafiltration of blood plasma within glandular acini, then traverses a network of collecting and draining ducts. Throughout this duct system, the primary saliva undergoes modifications, involving the reabsorption of specific substances and the secretion of others, ultimately leading to the production of final saliva. The final saliva is then transported into the oral cavity through the main excretory ducts.

Saliva secretion occurs in two distinct phases:

1. Ultrafiltration of blood plasma within the acini of the salivary glands, resulting in the creation of primary saliva within the acini lumen.

2. Reabsorption and selective secretion of particular substances as it travels through the intricate system of collecting and draining ducts within the salivary gland. This complex process culminates in the creation of final saliva, as depicted in Figure 2.2.

The process of secreting water and electrolytes in saliva involves their transfer from the blood plasma into the acinus lumen of the salivary gland. For this to occur, water and electrolytes must traverse the acinar cell, traversing both its basal and luminal membranes. As the membranes of all cells, including acinar cells, possess a lipid structure that renders them impermeable to polar molecules, specific protein channels become essential for the electrolyte transport. Channels designed for Na^+, K+, and Cl^- play a crucial role, facilitating the movement of these electrolytes through the acinar cell membrane in the direction of a lower concentration gradient without requiring energy expenditure.

However, when electrolytes move against the concentration gradient, from a lower to a higher concentration, specific transport systems come into play: Na^+-ATPase and K+-ATPase. In this scenario, energy is required for the movement of Na^+ and K^+, and this energy is derived from ATP. This type of substance transport through the cell membrane is termed active transport.

Under the influence of sympathetic and parasympathetic transmitters (mediators), a secretory potential of -30mV is established on the membrane of the acinar cell. The negative sign of the secretory potential is a result of membrane depolarization, driven by the entry of Na^+ ions into the acinar cell and the exit of K^+ ions from the acinar cell. The movement of Na^+ ions is guided by the concentration gradient of this cation, moving from an area of higher concentration (interstitium) into the acinar cell where its concentration is lower. Similarly, K^+ ions move in the opposite direction, from the acinar cell (with higher concentration) to the interstitium (with lower concentration). This leads to a change in electrification, causing the acinus membrane to become electronegative on the outside, resulting in a negative sign for the secretory potential.

The entry of Na^+ cations is invariably accompanied by the penetration of Cl^- anions. The subsequent destiny of Na^+, the primary cation in extracellular fluids, unfolds as follows: it is expelled from the acinar cell through the Na^+-pump transport system, utilizing energy derived from ATP (Na^+-ATPase). Energy from ATP is essential as Na^+ now moves against the concentration gradient, from a region of lower concentration to one of higher concentration. Through the action of the Na^+-pump, Na^+ is ejected into the lumen of the acinus. This expulsion of Na^+ is followed by the movement of Cl-. Simultaneously, water exits the acinar cell through osmosis via specific water channels situated on the apical, i.e. lumenal part of the acinar cell membrane. These orchestrated processes result in the formation of primary saliva within the acinus lumen, which is isotonic in comparison to blood plasma.

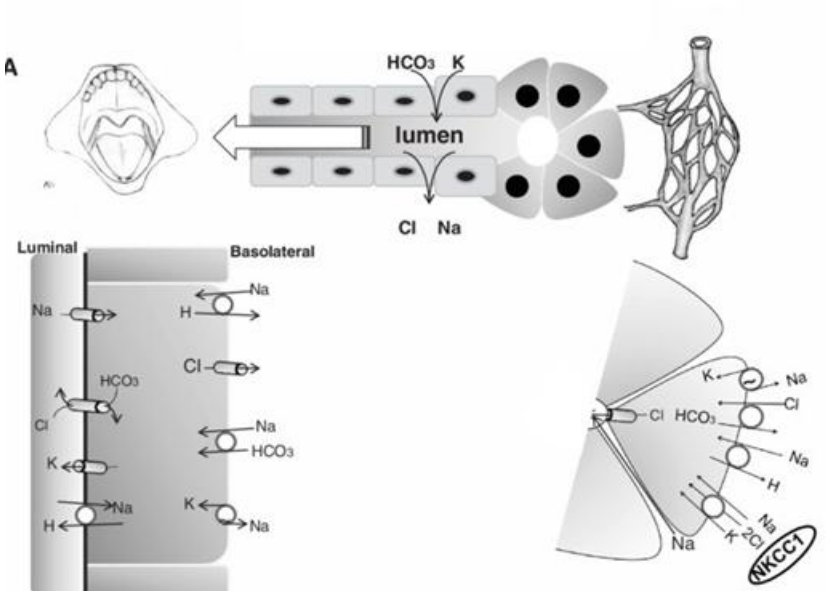


Figure 2.2: *Mechanism of salivary secretion.*Salivary glands comprise highly *specialized epithelial cells, known as acinar cells, with a clear division into two functional and anatomical regions: acinus and ductus (A). Within the acinar cell, water and electrolytes traverse from the interstitium. The ductal cells play a crucial role in reabsorbing Na and Cl while secreting K and HCO_3 (A-C). Upon Ca activation of both lumenal and basolateral transport compartments of the cell, a substantial volume of isotonic NaCl-rich fluid is produced within the acinus lumen. The primary function of salivary gland duct cells is to modify the initially isotonic saliva formed in the acinus lumen. Due to the reabsorption of Na^+ and Cl^- surpassing the secretion of K^+ and HCO_3^- the final saliva becomes hypotonic (C).*

The transport of water and electrolytes in the salivary glands is significantly facilitated by specific proteins known as aquaporins (AQPs). These proteins form a family with the primary function of transporting water across cell membranes. In the salivary glands, three classes of aquaporins are present: AQP-1, AQP-5, and AQP-8. AQP-1 is found in the microvascular endothelial cells of the salivary glands, AQP-5 is located on the apical membrane of the acinar cell, and AQP-8 is situated on the basolateral membrane of the acinar cell. Among these, AQP-5 has been established as the most crucial for water transport through the cell membrane (Figure 2.3).

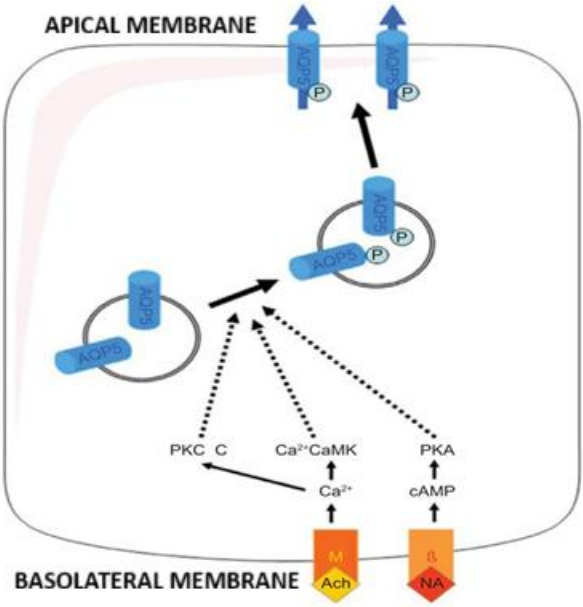


Figure 2.3: *Three classes of aquaporins: AQP-1, AQP-5, and AQP-8, which play an important role in the transport of water and electrolytes across the acini cell*

Primary saliva, concerning electrolyte concentration, is isotonic with blood plasma and constitutes its ultrafiltrate. It maintains a consistent composition, with a Na^+ concentration of 146 mmol/l and a K^+ concentration of 4 mmol/l. The composition of primary saliva remains uniform irrespective of whether it is produced through sympathetic or parasympathetic stimulation. The only distinction lies in the fact that parasympathetic stimulation allows for a more pronounced secretion due to increased interstitial circulation within the salivary gland.

During the transit of primary saliva through the network of collecting and excreting ducts (Figure 2.4) in the salivary gland, electrolytes undergo reabsorption, returning them to the blood plasma. As a result, final saliva becomes hypotonic compared to blood plasma. In this process, the reabsorption of Na^+ takes precedence over K^+ (Na^+ is reabsorbed, while K^+ is secreted). This is facilitated by Na^+-ATPase and K+-ATPase, utilizing energy from ATP. The Cl^- ion passively follows the movement of Na^+, necessitating the establishment of a balance between cations and anions during the formation phase.

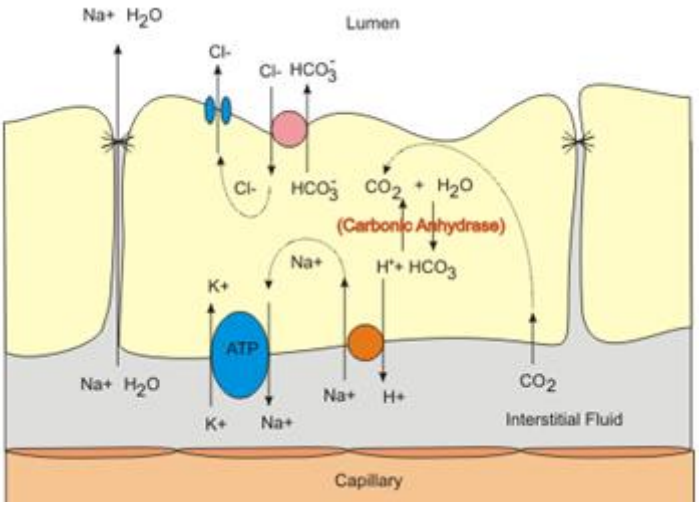


Figure 2.4: *The interchange of potassium and sodium occurs as primary saliva traverses the system of collecting and draining ducts (illustrated in the figure, depicting the events in a duct cell).*

The process of bicarbonate secretion mirrors the chloride secretion process and is linked to an elevation in bicarbonate concentration within the

acinar cell. The entry of these ions into the acinar cell is facilitated by carbonic anhydrase and relies on the exchange of Na^+ and H^+ ions, along with the Na^+ gradient (refer to Figure 2.5). Bicarbonate ions (HCO_3^-) access the acinar cell through calcium-dependent Cl^- channels.

Ductal cells also secrete bicarbonate ions, but reabsorption occurs during the transit through the system of collecting and excretory ducts, resulting in low concentrations of these ions in unstimulated saliva. During stimulated salivary secretion, the swift flow of primary saliva through the duct system leads to minimal reabsorption of bicarbonate ions. This accounts for the heightened concentration of bicarbonate ions in stimulated saliva.

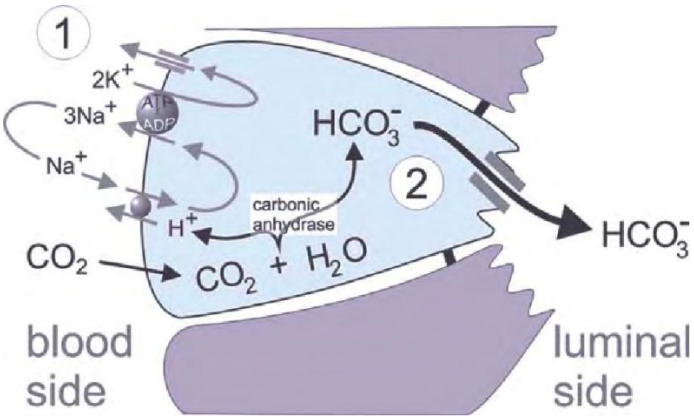


Figure 2.5: The process of water and electrolyte secretion involves the conversion of carbon dioxide inside cells to HCO_3^- and H^+ by carbonic anhydrase. HCO_3^- is secreted through the apical membrane of the cell via an anion channel (refer to 2). Simultaneously, H^+ is actively expelled through the basolateral membrane with the assistance of Na^+/H^+ exchange, wherein it gains energy from the Na^+ gradient formed due to the activity of the $Na^+/K+$ ATPase (indicated as 1). The elimination of protons from the cell is crucial for carbonic anhydrase to generate HCO_3^-.

The acinar cells of the salivary glands are responsible for secreting macromolecules, water, and electrolytes, whereas the ductal cells specialize in reabsorbing electrolytes. Glandular cells that are inserted, i.e. intercalated (positioned between acinar and ductal cells) share more functional similarities with acinar cells than with ductal cells. These cells secrete a low concentration of proteins but play a significant role in the secretion of water and bicarbonates.

Calcium and phosphate secretion

Saliva contains calcium and phosphates, crucial for preventing tooth demineralization. There is a unidirectional transport of these electrolytes from the acinar cell into the acinar lumen. The acinar cell possesses all the necessary components for calcium ion translocation. Ca^{2+} enters the acinar cell through the basolateral membrane, traverses the channels of the endoplasmic reticulum, and exits the acinar cell through the apical membrane. Similarly, phosphate ions enter the acinar cell through Na^{+} channels and exit through the apical membrane of the acinar cell (Figure 2.6). The movement of phosphates is contingent on the concentration gradient of these ions within the acinar cell.

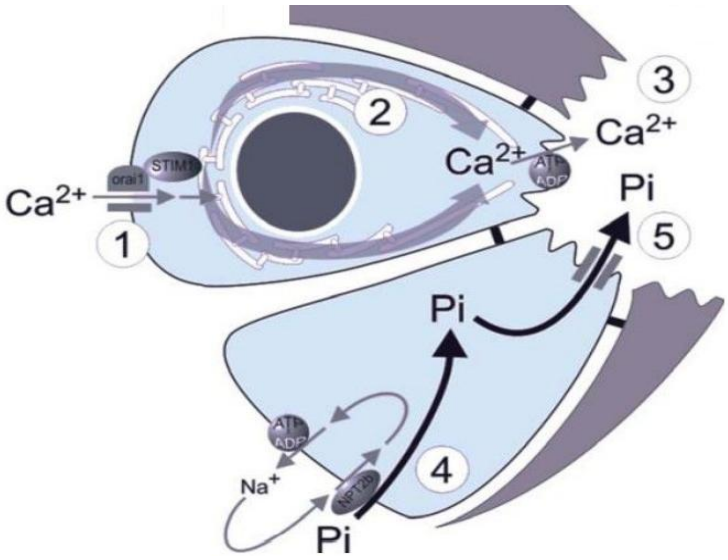


Figure 2.6: *A potential mechanism for Ca^{2+} translocation involves its entry through the basolateral membrane via Orai1 Ca^{2+} channels (1). Subsequently, it traverses the cell via the endoplasmic reticulum (2) before being released at the apical side and extruded by the PMCA (Plasma Membrane Calcium ATPase) (3). In the active step of phosphate translocation, phosphate ions are taken up across the basolateral membrane by the Na^{+}-dependent phosphate transporter NPT2b. This process leverages the reverse Na^{+} gradient to concentrate phosphate inside cells. Phosphate exits across the apical membrane through a mechanism that is yet to be determined.*

The composition of primary and final saliva varies markedly, primarily in terms of electrolyte content and osmotic concentration. Final saliva is characterized by its hypotonic nature, with an osmolality one-tenth that of primary saliva, equivalent to the osmolality of blood serum. Notably, final saliva exhibits a heightened concentration of potassium (K^+). Potassium, predominantly found as the main cation in intracellular fluids, typically maintains a lower concentration in extracellular fluids. However, saliva deviates from this norm, representing the body fluid with the highest potassium content. This elevated potassium concentration is attributed to the substitution of sodium for potassium (Na^+ reabsorption, K^+ excretion) within the collecting and excretion ducts of the salivary glands.

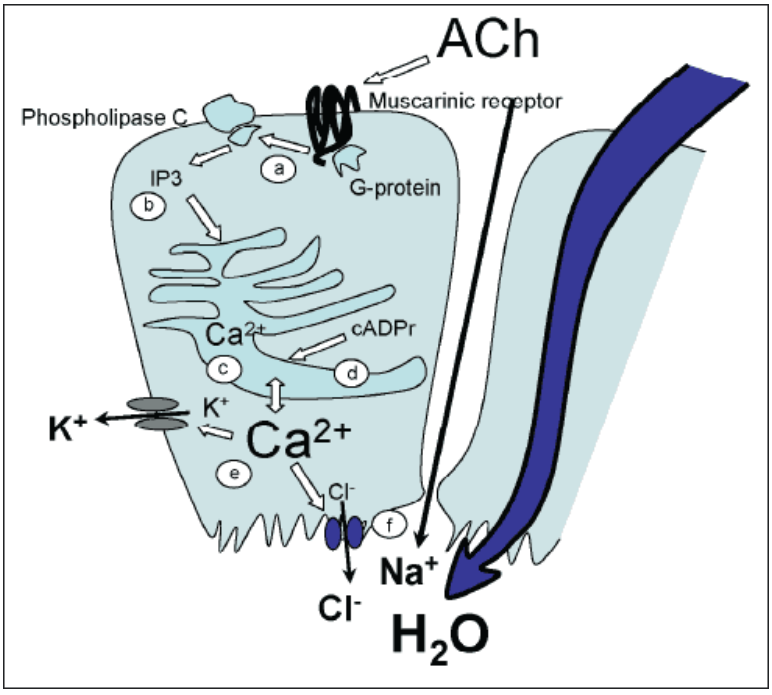


Figure 2.7: The role of acetylcholine in the regulation of salivary electrolyte secretion

The concentration and electrolyte ratio in saliva exhibit notable variations depending on the intensity of stimulation and the salivary flow rate. In instances of insufficient secretion or when there is an absence of stimulation, unstimulated saliva is notably hypotonic. This hypotonicity arises from the slow movement of secretion through the network of collecting and excretion ducts, facilitating nearly complete reabsorption of

electrolytes from the primary secretion. Conversely, during heightened secretion levels (stimulated saliva), the osmolality of saliva can approach three-fourths of the osmolality of blood serum. This increase is attributed to the inability of the collecting and excretion ducts within the salivary glands to fully reabsorb electrolytes during the rapid flow of primary saliva. Consequently, a significant portion of electrolytes is retained in the final secretion, resulting in heightened osmolarity of final saliva.

The regulation of water and electrolyte secretion by the salivary glands involves the crucial involvement of secondary messengers such as inositol-3-phosphate (IP_3) and Ca^{2+}. The concentration of calcium within the cell's cytoplasm, primarily stored in the endoplasmic reticulum of the acinar cell) is very low (<100 nM), so even a minimal increase in its concentration can act as an intracellular signal. As a response to various stimuli in the acinar cell itself, the Ca^{2+} ion is released from its intracellular reserves. The release of Ca^{2+} is supported by the penetration of Ca^{2+} from the extracellular environment into the interior of the acinar cell.

The parasympathetic neurotransmitter, acetylcholine, elicits an elevation in the concentration of intracellular Ca^{2+} within the acinar cell, as depicted in Figure 2.7. Specific receptors located on the acinar cell membrane bind acetylcholine, initiating the activation of the G-protein embedded in the membrane itself. Subsequently, the G-protein stimulates the membrane enzyme phospholipase-C. This enzyme facilitates the breakdown of the membrane lipid phosphatidyl-inositol into diglyceride and inositol-3-phosphate (IP_3). Serving as a secondary messenger, inositol-3-phosphate induces an increase in intracellular Ca^{2+} concentration by liberating it from intracellular stores. It binds to (IP_3)-receptors situated in the endoplasmic reticulum, triggering a substantial release of Ca^{2+} from its reservoirs in the endoplasmic reticulum, as illustrated in Figure 2.8. Furthermore, calcium, acting as a secondary messenger, activates chloride channels, leading to an augmentation in the secretion of water and electrolytes by the salivary glands.

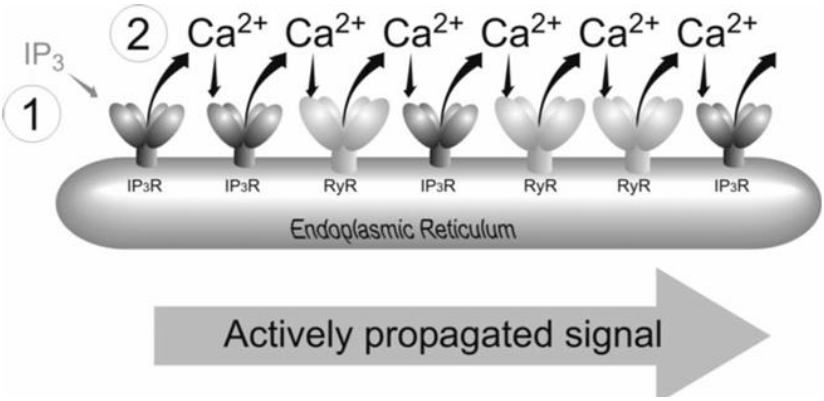


Figure 2.8: IP_3 initiates the release of calcium ions (Ca^{2+}) from IP_3 receptors (IP_3R). The Ca^{2+} already released autonomously stimulates additional Ca^{2+} release from both IP_3R and RyR receptors. The release of Ca^{2+} from one receptor triggers the activation of the subsequent receptor, leading to active signal propagation. Consequently, the Ca^{2+} signal can initiate in the apical part of the cell and rapidly propagate throughout the entire cell.

The transfer of both organic and inorganic substances from blood plasma to saliva occurs through various mechanisms, as illustrated in Figure 2.9:

1. Ultrafiltration through the microspaces between acinar cells.
This mechanism facilitates the transport of molecules with a molecular mass of less than 1900 Da, encompassing substances such as water, ions, hormones, catecholamines, and certain other hormones.

2. Filtration through the pores of the cell membrane of acinar cells:
Limited to molecules with an exceptionally low molecular mass, specifically less than 400 Da, this mode of transport predominantly involves substances like water.

3. Transport across the cell membrane of the acinar cell:

- passive diffusion exclusive to lipophilic molecules (steroid hormones, medicines, certain drugs)
- active transport through protein channels
- pinocytosis (refers to larger protein molecules in aqueous solution)

4. Transport through specific ion channels. This method of transportation is specifically constrained to certain electrolytes (Na^+, K^+, Cl^-, Ca^{2+}).

5. There is the transudation of certain blood plasma components through the gingival sulcus or directly through the oral mucosa, notably exemplified by albumin.

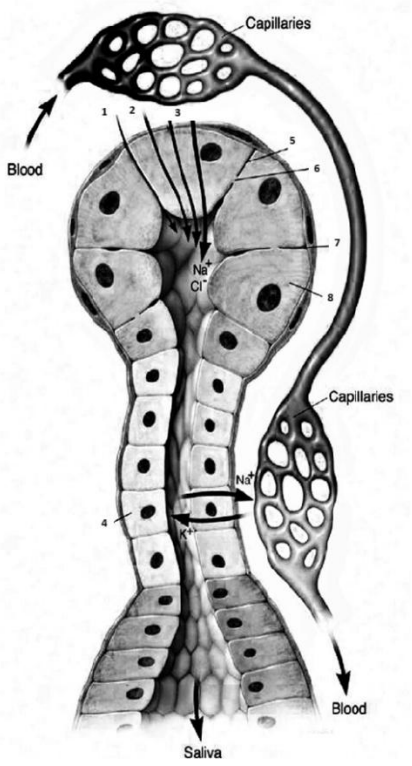


***Figure 2.9**: Mechanism of transport of proteins and ions from the blood plasma into the salivary ducts. a. active transport, b. passive diffusion, c. ordinary filtration 4. Na^+ of ductal cells 5. cell membrane 6. pore, 7. intercellular space 8. acinar cell*

BIOCHEMICAL COMPOSITION OF SALIVA

In terms of quantity, water comprises the majority of saliva at 99%. The remaining 1% consists of various components, including:

- organic molecules such as proteins, glycoproteins, lipids, and carbohydrates (e.g., glucose).
- final metabolites such as urea, uric acid, creatinine, and bilirubin contribute
- steroid hormones;
- drugs and their metabolites;
- inorganic ingredients-electrolytes (Na^+, K^+, Cl^-, Ca^{2+}, phosphates, bicarbonates);

The majority of organic components in saliva are generated by the acinar cells within the salivary glands. Certain components are synthesized by ductal cells, while others are derived from the blood plasma.

Saliva comprises of various components that originate from blood plasma, including:

- Water,
- Electrolytes,
- Separate proteins (albumin),
- Glucose,
- Vitamins,
- Steroid hormones,
- Drugs and their metabolites,
- Final metabolites (urea, uric acid, creatinine).

The components of saliva originating from the salivary glands include peptides, proteins, and glycoproteins. The group of saliva proteins and glycoproteins encompasses:

a) Enzymes: salivary amylase, salivary lipase, oral peroxidase, lysozyme, catalase, superoxide dismutase, glutathione peroxidase, glutathione reductase

b) Other proteins and glycoproteins of saliva: cystatins, growth factor, fibronectin, histatins, defensins, sialomucin, cathelicidins, proteins rich in proline, statherin, immunoglobulins

The concentration of proteins in saliva is notably lower compared to their levels in blood serum, as indicated in Table 2.1. While certain salivary proteins are present in very low concentrations, others, such as salivary amylase, proline-rich proteins, sialomucin, and secretory immunoglobulins, are present in significantly higher concentrations in saliva.

Table 2.1: Differences in the concentration of individual organic and inorganic components between saliva and blood plasma

Components	Saliva	Blood plasma
Inorganic components (mmol/L)		
Ca^{2+}	1-2	2,5
Mg^{2+}	0,2 - 0,5	1,0
Na^{+}	6-26	140
K^{+}	1-7	4
NH_4^{+}	14-32	0,03
H_2PO_4 and HPO_4^{2-}	2-23	2
Cl^{-}	17-29	103
HCO_3^{-}	2-30	27
F-	0,0005-0,005	0,001
SN^{-}	0,1-2,0	-
Organic components (mmol/L)		
Urea	2-6	5
Uric acid	0,2	0,3
Free amino acids	1-2	3-5
Glucose	0,05	5
Lactate	0,1	1
Fatty acids (mg/L)	10	3.000
Macromolecules (mg/L)		
Proteins	1.400-2.000	70.000
Glycoproteins	110-300	1.400
Amylase	380	-
Lysozyme	109	-
Peroxidase	3	-
Iga	194	1.300
Igg	14	13.000
Igm	2	1.000
Lipids	20-30	2.000

Observations from Table 2.1 reveal that a majority of components originating from the blood and present in saliva exhibit significantly lower concentrations compared to their levels in blood plasma. Notably, potassium ions (K^+) stand out as an exception, with their concentration in saliva being notably higher than in blood. Furthermore, specific components derived from acinus and duct cells, such as salivary amylase, proline-rich glycoproteins, and sialomucin, are exclusive to saliva and are absent in physiological conditions in blood plasma.

Saliva and perception of taste stimuli

The primary saliva, originating in the acinus lumen, maintains isotonicity with blood plasma based on electrolyte concentrations. As the primary saliva traverses the network of collecting and draining ducts, there is a notable shift in electrolyte concentrations. Specifically, sodium (Na^+) and chloride (Cl^-) undergo near-complete reabsorption, and a significant proportion of $HCO3^-$ ions is reabsorbed. Conversely, as the primary saliva progresses through the collecting and draining ducts, there is an excretion of potassium ions (K^+) (see Figure 2.10).

In the context of salivary physiology, the pertinence of final saliva exhibiting hypotonicity (equivalent to 1/6 of the osmolarity of blood plasma) becomes a subject of consideration. This hypotonic state assumes significance in the facilitation of taste stimuli perception. If salivary electrolyte concentrations, specifically sodium (Na^+) and chloride (Cl^-), mirrored those of blood plasma, the sensitivity to low concentrations of dietary salt would be compromised. The nuanced regulation of taste perception is particularly evident during unstimulated salivary secretion, where not only are sodium and chloride concentrations diminished, but glucose, bicarbonate, and urea concentrations also register lower levels in unstimulated saliva. Moreover, a discernible variance exists between the concentrations of these biomolecules in unstimulated saliva and blood plasma, with the former aligning closely with the requisite threshold for sensory detection. A notable distinction is observed notably between sodium and chloride concentrations in saliva and blood plasma, surpassing the threshold essential for the detection of salty tastes. This observation is expounded further in Table 2.2, which delineates the compositional ratio between plasma and unstimulated saliva, elucidating the perceptual nuances associated with individual tastes (all concentrations are denoted in mmol/L).

Table 2.2: Difference in concentration of certain components in blood plasma and saliva related to taste perception

mmol/L	Na	Sour Cl	H	HCO_3	Sweet glycose	Bitter urea
Blood	145	101	$4x10^{-5}$	24	4.5	6
Saliva	6	16	$1x10^{-4}$	5	0.08	4
Taste perception threshold	NaCl	HCl		$NaHCO_3$	sucrose	urea
	12	0.8		10	30	90

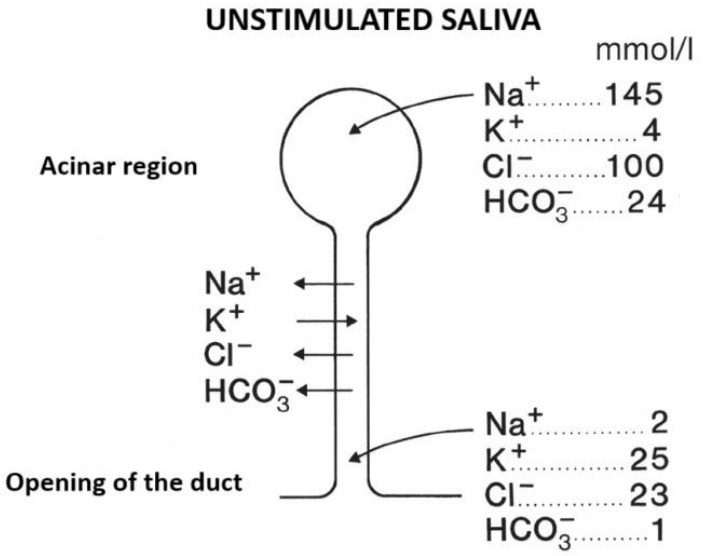


Figure 2.10: *Difference in electrolyte concentrations of primary and final unstimulated saliva*

Calcium and phosphate concentration in saliva

The salivary composition includes a saturation of calcium, phosphates, and hydroxyl ions, integral in forming the solid dental structure. These ions play a dual role: they contribute to the formation of hard mineral deposits on teeth, commonly known as tartar, while concurrently serving as a protective barrier against erosion and caries.

Saliva exhibits a calcium concentration lower than that of blood plasma (Ca = 2.5 mmol/L) but a higher concentration of phosphates (inorganic P = 1 mmol/L) (Table 2.1). The mechanisms underpinning the elevated phosphate concentration in saliva, in contrast to blood plasma, remain elusive. Notably, diverse salivary secretions from distinct glands present varying calcium and phosphate concentrations. Parotid saliva, for instance, displays lower calcium and higher inorganic phosphate levels compared to submandibular saliva. Saliva originating from minor mucous glands is further characterized by an exceptionally low phosphate concentration (0.4 mmol/L).

By enhancing saliva secretion, there is a corresponding reduction in the concentration of total phosphates within saliva, accompanied by an elevation in saliva pH and the overall concentration of bicarbonates. The rise in saliva pH instigates alterations in the concentrations of distinct pH-dependent forms of salivary phosphates, namely dihydrogen phosphate ($H_2PO_4^{1-}$), monohydrogen phosphate (HPO_4^{2-}), and phosphate (PO_4^{3-}). Specifically, the concentration of dihydrogen phosphate ($H_2PO_4^{1-}$) diminishes, while the concentration of monohydrogen phosphate (HPO_4^{2-}) experiences a slight increase. Notably, there is a substantial increase in the concentration of phosphate (PO_4^{3-}) (Figure 2.11). This pronounced elevation in phosphate (PO_4^{3-}) concentration holds significant implications for preserving the structural integrity of solid dental substances.

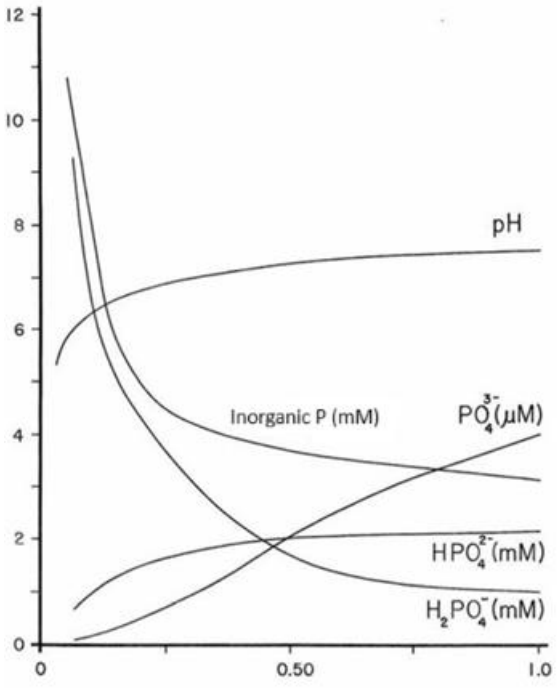


Figure 2.11: Display of the concentration of different forms of phosphate depending on the salivary flow rate

Upon examining the electrolytes, it becomes apparent that their concentrations rise with an augmented secretion of saliva. Notably, the concentrations of electrolytes integral to the crystal lattice of solid dental substances—specifically, calcium ions (Ca^{2+}), phosphate ions (PO_4^{3-}), and hydroxyl ions (OH^-)—demonstrate an increase. This phenomenon plays a pivotal role in the remineralization processes essential for maintaining tooth integrity. However, it also introduces the potential for the accumulation of more substantial hard deposits on the teeth.

3 SALIVA PROTEINS

GENERAL CHARACTERISTICS OF SALIVA PROTEINS

MECHANISM OF SECRETION OF SALIVA PROTEINS

GENERAL CHARACTERISTICS OF SALIVA PROTEINS

The composition of saliva comprises a wide range of proteins with diverse biological functions. The majority are synthesized by the salivary glands, while others—such as albumin, certain immunoglobulin classes, and hormones—are derived from blood plasma.

Salivary proteins play several critical roles in maintaining oral homeostasis, including digestive activity, antimicrobial defense, regulation of electrolytes, microbial aggregation, protection of tooth enamel, lubrication, and buffering capacity. Their "biological pathway" begins with intracellular biosynthesis, followed by post-translational modifications such as glycosylation, phosphorylation, proteolysis, and sulfation, culminating in their secretion into the oral cavity. Salivary proteins are highly dynamic and variable biomolecules, with their variability influenced by the timing of synthesis, their intrinsic biochemical properties, and various external and internal factors that may alter their structure or function.

The field of research focused on identifying and characterizing various classes of salivary proteins has advanced significantly in recent years. To date, over 3,000 distinct proteins have been identified in human saliva. Among the most prominent families of salivary proteins are proline-rich proteins (PRPs), histatins, statherins, cystatins, and α-amylase. In addition to these salivary-specific proteins, this oral fluid also contains proteins commonly found in other biological fluids (Table 3.1). These include mucins, lysozyme, lactoferrin, peroxidase, carbonic anhydrase, salivary immunoglobulin A (IgA), immunoglobulin G (IgG), albumin, defensins, cathelicidins, and others.

Approximately 20% of the total salivary protein content is also present in serum, which underscores the relevance of salivary protein alterations—both quantitative and qualitative—in association with various disease states. This evidence highlights the potential of saliva to serve as a non-invasive diagnostic fluid, not only for oral pathologies but also for selected systemic diseases and conditions.

There are certain challenges in the classification of these biomolecules, which has led to the presence of various salivary protein classification systems in the literature. These classifications are primarily based on the functional roles of the proteins and their distribution across body fluids.
The group of proteins and glycoproteins originating from the salivary glands includes:

➤ Enzymes: amylase, lipase, salivary peroxidase, lysozyme, catalase, superoxide dismutase, glutathione peroxidase, and glutathione reductase.
➤ Other salivary proteins and glycoproteins: cystatins, growth factors, fibronectin, histatins, defensins, mucins, cathelicidins, proline-rich proteins, statherin, and immunoglobulins.

The total protein content in saliva ranges from 0.5-2.5 g/l. According to their distribution in body fluids, salivary proteins can be classified into three groups (Table 3.1):

I. group: proteins found exclusively in saliva
(histatins and acidic proline-rich proteins);

II. group: proteins found in several different mucous secretions, which according to their structural and biochemical characteristics are very similar although they are produced by different glands (mucin, lysozyme);

III. group: proteins that are not products of the salivary glands
(some immunoglobulins and albumin);

Table 3.1: Distribution of salivary proteins in different body fluids

Class	saliva	tears	nasal secretion	bronchial secretion	seminal (amniotic) fluid	mucous uterine secretion	sweat	plasma
Mucins	++++	+	++++	++++	+	++++	++++	-
Acidic PRP	++++	-	-	-	-	-	-	-
Alpha amylase	++++	+	+	+	+	+	+	+
Basic PRP	+++	-	+	+	-	-	-	-
Secretory IgA	+++	++++	++++	-	+	-	+	
Cystatins	++	+	+	-	+	+	+	++
Statherin	++	+	+	+	-	-	-	-
IgG	+	+	+	+	+	-	+	++++
EP-GP	+	+	+	-	+	-	+	-
Histatins	+	-	_	_	_	_	_	_
Lysozyme	+	++++	+++++	++++	+	+	-	+
Kallikrein	+	-	+	-	+	-	+	+
Lactoferrin	+	++++	+++++	++++	+	+	-	+
Peroxidase	+	+	+	-	-	+	-	-
IgM	+	+	+	+	-	-	-	++
Albumins	+	+	+	+	-	+	+	++++

- (not detected), + (< 1% of total protein), ++ (between 1 and 5% of total protein), +++ (between 5 and 15% of total protein), ++++ (> 15% of total protein)

Peptides, proteins, and glycoproteins derived from the salivary glands are synthesized by both acinar and ductal epithelial cells. The three pairs of major salivary glands differ in the composition and nature of their protein secretions (Table 3.2). Sublingual saliva, produced predominantly by mucous acinar cells, is rich in mucins—high molecular weight glycoproteins—which results in a highly viscous and thick secretion. In contrast, the serous acinar cells of the parotid gland primarily synthesize and secrete salivary amylase and proline-rich glycoproteins (PRPs). As a result, parotid saliva is less viscous and exhibits a thinner consistency.

Table 3.2. Concentration Ranges of Selected Salivary Proteins (µg/mL)

Protein	Parotid Secretion (Min – Max)	Submandibular/Sublingual Secretion (Min – Max)	Whole Saliva (Min – Max)
Amylase	650 – 2,600	0 – 0	380 – 500
Cystatin	2 – 4	92 – 280	240 – 280
Proline-Rich Proteins (PRP)	230 – 1,251	270 – 1,335	90 – 180
Mucins	0 – 0	80 – 560	90 – 700
Secretory IgA (sIgA)	20 – 230	41 – 56	19 – 439
Total Protein	**902 – 4,085**	**483 – 2,231**	**819 – 2,099**

Various stimuli—including mechanical, chemical, and others—not only influence the *volume* of salivary secretion but also modulate its *composition*. It has been well-documented that different types of stimulation elicit differential expression and secretion of specific classes of salivary proteins, both in whole (mixed) saliva and in secretions from the major salivary glands—namely, the parotid and submandibular/sublingual glands (SMSL) (see Table 3.3).

Table 3.3: Alterations in salivary protein composition in response to different stimuli

Saliva type	stimulant	changes in secretion
SMSL	fruit candies	Mucin, type-1 (+) Mucin, type-2 (NP) Lactoferrin (-) Peroxidase (-) Amylase(-) Carboanhydrase (+) PRP (+) PRP Basic and Glycosylated (+)
Total (mixed)	after toothbrushing	Albumin (+)
Parotid and SMSL	mechanical and acidic	Histatins, parotid (-) Histatins, SMSL (+)
Total (mixed)	citric acid stimulation	PRP (+) Alpha amylase (+) Calgranulin (+) Annexin (+) 9 unidentified proteins (+)
parotid	citric acid stimulation	IgA (-) Albumin (-) lysozyme (-) Amylase (NP)

(+) indicates an increase in concentration following stimulation; (–) indicates a decrease in concentration following stimulation; (NC) indicates no change following stimulation.

The minor salivary glands contribute approximately 8–10% to the total volume of mixed saliva. Although the quantity of saliva they produce is relatively small, these glands play a crucial role in oral physiology and the maintenance of oral homeostasis.

This significance is attributed to:

- their strategic distribution throughout the oral mucosa,
- their anatomical proximity to the surface of the oral epithelium,
- their high concentrations of mucins and immunoglobulins relative to the major salivary glands.

Mucin – The secretion of minor mucous glands contains two primary types of mucins: high-molecular-weight and low-molecular-weight forms. These macromolecules play a vital role in preventing dehydration of the oral mucosa, protecting it from mechanical injury, and shielding it from pathogenic microorganisms. Additionally, mucins form complexes with smaller salivary proteins such as secretory immunoglobulin A (sIgA), lysozyme, histatins, and proline-rich glycoproteins (PRPs). Through these complexes, mucins from minor salivary glands protect smaller salivary glycoproteins from proteolytic degradation by enzymes of various origins.

Cystatins – Several classes of salivary proteins known as cystatins (cystatins A, B, D, SN) have been identified in the mucous secretions of the minor salivary glands. Their presence indicates a role in protecting oral tissues from damage caused by cysteine protease enzymes. Specifically, cystatins function as inhibitors that inactivate these proteolytic enzymes.

Proline-Rich Glycoproteins (PRPs) – Although PRPs are primarily synthesized in the major salivary glands, five distinct classes have also been detected in the secretion of the minor salivary glands. These proteins are essential for maintaining mineral balance in saliva and for protecting the hard tissues of the teeth.

Histatins – In saliva originating from the minor mucous glands, two classes of histatins—histatin 3 and histatin 1—have been identified. These salivary proteins contribute to the innate immune defense of the oral cavity. Histatins exhibit fungicidal activity (e.g., against *Candida albicans*) and bacteriostatic effects by inhibiting the proliferation of *Streptococcus mutans*.

Recent studies have identified 12 novel salivary proteins present in the secretion of the minor labial mucous glands, making their secretion distinct and unique in terms of protein composition. The salivary proteins produced by minor mucous glands differ from those secreted by the major salivary glands based on the following two characteristics:

– protein analysis of the secretion of the small mucous glands shows the presence of only 56 different salivary proteins, while the secretion of the parotid, submandibular and sublingual glands proves the presence of more than a thousand salivary proteins,

– one quarter of the salivary proteins produced by the small mucous glands are characteristic of these glands and are not produced in the large salivary glands.

MECHANISM OF SECRETION OF SALIVARY PROTEINS

The synthesis of local salivary proteins occurs in the acinar and ductal cells of the salivary glands. The process begins in the ribosomes of the rough endoplasmic reticulum (ER), where the primary polypeptide chains are synthesized. These nascent proteins are then transported into the cisternae of the ER, from which they are subsequently conveyed to the Golgi apparatus. Within the Golgi apparatus, the carbohydrate moieties of salivary glycoproteins are synthesized and covalently attached to the polypeptide backbone in the form of side chains. Due to their high molecular weight, the resulting glycoproteins cannot freely diffuse across the plasma membrane. Instead, they are secreted into the lumen of the acinus via exocytosis.

The secretion of proteins and glycoproteins in the salivary glands takes place in several stages: synthesis, packaging, deposition, and release. Each of these stages is regulated by protein phosphorylation, which in turn is associated with cyclic adenosine monophosphate (cAMP). This compound is known as a secondary messenger in the mechanism of action of many hormones, that is, in the regulation of biochemical processes in cells. The increase in the level of cAMP in the acinar and ductal cells stimulates all stages involved in the process of synthesis and secretion of salivary proteins. Therefore, the control of the secretion and release of local salivary proteins depends on the level of cAMP in the cells of the salivary glands.

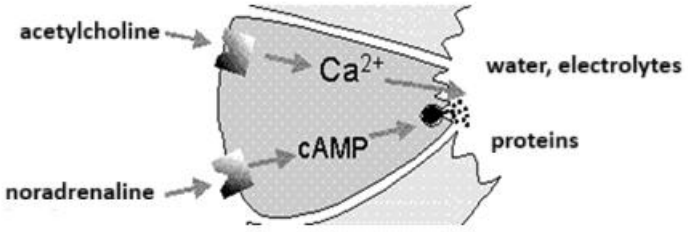


Figure 3.1: *Secretion of water, electrolytes, and proteins in the acinar cells of the salivary glands. Proteins are secreted by exocytosis, which is under sympathetic control and involves noradrenaline and cAMP.*

β-adrenergic receptors, located on the membranes of acinar and ductal cells, mediate the stimulation of protein synthesis and secretion within the salivary glands. The binding of the hormone noradrenaline to these receptors activates the membrane-associated G-protein in acinar cells. This activation triggers the enzyme adenylyl cyclase, which catalyzes the conversion of ATP to cyclic adenosine monophosphate (cAMP). Acting as a secondary messenger, cAMP activates protein kinase A enzymes, which subsequently phosphorylate the newly synthesized proteins (Figure 3.1).

Activation by adrenergic nerves is not the sole mechanism stimulating protein synthesis in acinar and ductal cells. In addition to adrenergic and cholinergic innervation, the salivary glands also receive input from peptidergic neurons. Among these, vasoactive intestinal peptide (VIP) is present. This peptide also causes stimulation of salivary protein secretion by increasing cAMP levels in salivary gland cells.

Almost all salivary proteins or peptides can be classified functionally as **antimicrobial peptides (AMPs).** The majority of AMPs are cationic molecules carrying positive charges. Nonetheless, a smaller subset of anionic (negatively charged) AMPs is also present in saliva. Despite being grouped together based on their antimicrobial function, the increasing identification of novel AMPs complicates their unified classification and characterization. Their primary structures vary widely, ranging from single peptide chains of 10 to 50 amino acids to larger peptides comprising several hundred amino acids. Cationic AMPs are characterized by the presence of positively charged amino acid residues, such as arginine, lysine, histidine, and a significant proportion of hydrophobic residues.

Based on their secondary structure, antimicrobial peptides in saliva are classified into four groups (Figure 3.2):

1. peptides with an extended α-helix structure
2. peptides with numerous disulfide bonds that connect in a β-direction
3. peptides with single disulfide bonds enabling cyclic repetition of peptide sequences,
4. extended peptides predominantly composed of amino acids

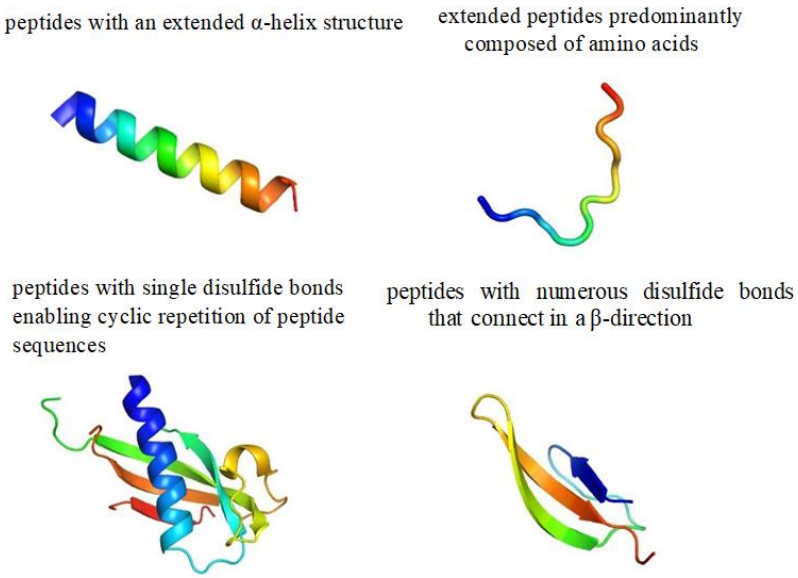


Figure 3.2: *Secondary structure of antimicrobial peptides from saliva*

Most AMPs are initially presented as prepropeptide precursors, which further undergo proteolytic modifications at the N-terminal portion to ultimately become active peptides. The majority of salivary proteins are secreted continuously into the oral cavity; however, certain proteins, such as immunoglobulin A (IgA), are secreted in response to specific stimuli.

The complexity in classifying salivary proteins arises from the fact that their definitive structural conformations often become apparent only upon biological interaction with various cellular membranes.

Antimicrobial salivary peptides possess both hydrophobic and hydrophilic domains. The positively charged residues of these peptides bind to lipopolysaccharides (LPS) in the cell membranes of gram-negative bacteria and integrate into the hydrophobic interior of the membrane. The subsequent mechanisms by which salivary peptides induce bacterial cell death vary among different peptides and remain incompletely elucidated.

A substantial number of salivary proteins play crucial roles in the immunological defense of the oral cavity. Certain defensive salivary proteins, such as salivary immunoglobulins and chaperone proteins, participate in both innate and adaptive immune responses. Cationic peptides and other salivary proteins, including lysozyme, amylase, proline-rich glycoproteins (PRPs), cystatins, mucins, peroxidase, and statherin, primarily mediate innate immune protection within the oral environment. Additionally, some of these proteins possess immunostimulatory and/or immunomodulatory properties.

Most salivary proteins are present in the oral environment at low concentrations. Despite their limited abundance, each salivary protein plays a vital role in maintaining the health of oral tissues. Many salivary proteins act synergistically with others, producing cumulative effects that establish an effective defense mechanism at the level of the oral mucosa.

Five (5) distinct defensive effects of salivary proteins have been identified in whole mixed saliva. These include:

- Microbial agglutination. Such effect demonstrate the proteins capable of binding to bacterial cell walls, other oral surfaces, or other proteins.
- Bacteriolysis, referring to the destruction of bacteria, primarily exerted by cationic salivary peptides and lysozyme.
- Fungicidal activity (involving the destruction of fungal organisms).
- Antiviral activity (preventing the replication of viral particles).
- Immunological effects, predominantly involving salivary immunoglobulins and other proteins that activate or modulate immune defense within the oral cavity.

Regardless of the various classifications found in the literature, for didactic purposes and to facilitate understanding of the role of salivary proteins in oral physiology and homeostasis, the following text categorizes them into three groups:

1. Salivary antimicrobial peptides, encompassing peptides present in saliva with a predominant antimicrobial function.
2. Other salivary proteins involved in the innate immune protection of the oral cavity.
3. Salivary proteins participating in the adaptive immune protection of the oral cavity

4 ANTIMICROBIAL PEPTIDES PRESENT IN SALIVA

LYSOZYME

Bacteriolytic effect of lysozyme

LACTOFERRIN

Antibacterial effect of lactoferrin

Antiviral effect of lactoferrin

Fungicidal effect of lactoferrin

HISTATINS

Formation of dental pellicle

Antibacterial and fungicidal properties of histatins

Binding of metal ions

Inhibition of bacterial and tissue proteolytic enzymes and mediators implicated in the pathogenesis of periodontal disease

Binding of polyphenols within the oral milieu

Promotion of wound healing

DEFENSINS

CALPROTECTINS

CATHELICIDINS

ADRENOMEDULLIN

PROTEINS THAT INCREASE THE PERMEABILITY OF THE BACTERIAL CELL MEMBRANE-BPI (BACTERICIDAL/PERMEABILITY INCREASING PROTEIN)

ORAL BIOCHEMISTRY AND PHYSIOLOGY

LYSOZYME

This salivary enzyme-protein plays a key role in nonspecific humoral antibacterial defense. It consists of a single polypeptide chain composed of 129 amino acids. Within the polypeptide chain, four intramolecular disulfide bonds are formed between cysteine residues at positions 6–127, 30–115, 64–80, and 76–94. The active site of lysozyme comprises six domains—A, B, C, D, E, and F. These domains collectively bind six sugar residues of peptidoglycans found in the bacterial cell wall. The primary catalytic groups at the active site originate from the amino acids glutamine and aspartic acid.

Based on its biochemical properties, lysozyme is classified as a basic glycoprotein, characterized by a positive charge and cathodic electrophoretic mobility, with an isoelectric point of 10.5. Its molecular weight is approximately 14,300 Da.

Lysozyme is secreted by both major and minor salivary glands, as well as by leukocytes and gingival fluid.

Table 4.1: Lysozyme concentration in saliva (mg/ml)

	Mixed saliva		Parotid saliva		Submandibular-sublingual saliva
	Stimulated	Unstimulated	Stimulated	Unstimulated	Stimulated
Lysozyme	5-50	100-200	1-20	10-80	10-70

The concentration of lysozyme varies depending on the type of salivary secretion. It is found to have the lowest concentration in parotid saliva. Its levels are significantly higher in unstimulated salivary secretion compared to stimulated secretion (Table 4.1). Interestingly, women exhibit a higher salivary concentration of lysozyme than men.

Lysozyme plays multiple important roles in the nonspecific antibacterial defense of the oral environment:

- Bacteriolytic action
- Inactivation of many viruses
- Activation of phagocytosis by neutrophil granulocytes and macrophages
- Stimulation of the antitumor effects of monocytes
- Anti-inflammatory action
- Maintenance of the integrity of the cell membrane

Bacteriolytic action of lysozyme

Lysozyme catalyzes the hydrolytic cleavage of β (1,4) glycosidic bonds in N-acetylmuramic acid, a key component of proteoglycans and glycosaminoglycans in the bacterial cell wall. This enzymatic activity leads to bacterial cell lysis.

The natural substrate for lysozyme is murein, which is why this salivary protein is also termed muramidase. Murein, a peptidoglycan, constitutes a major structural element of the bacterial cell wall.

Gram-positive bacteria possess a thick cell wall composed of approximately 40 layers of peptidoglycan, within which chains of teichoic and lipoteichoic acids are embedded (Figure 4.1). In contrast, Gram-negative bacteria have a single layer of peptidoglycan that lacks teichoic acids. This peptidoglycan layer is enclosed by an asymmetric, double-layered outer membrane. The structural differences between Gram-positive and Gram-negative bacteria underlie their varying susceptibility to lysozyme. Gram-negative bacteria exhibit greater resistance to lysozyme due to the protective barrier provided by the outer membrane.

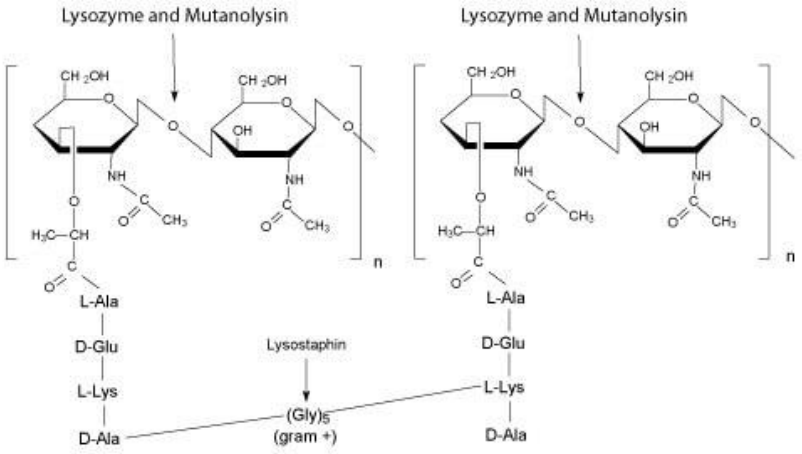


Figure 4.1: *The action of lysozyme on peptidoglycans of gram-positive bacteria (breaks the 1,4 glucosidic bond)*

In addition to the enzymatic bacteriolytic activity, lysozyme's non-enzymatic antibacterial effects have also been reported, although the exact mechanism remains incompletely understood. Due to its cationic properties, lysozyme is believed to bind to teichoic and lipoteichoic acids present in the bacterial cell wall. Upon binding, these polyanionic molecules may directly activate bacterial autolysins, which in turn induce bacterial cell lysis.

Another proposed mechanism for lysozyme's action is its ability to induce the release of divalent cations (Ca^{2+}, Mg^{2+}) from the bacterial cell membrane. These cations are known to play a crucial role in stabilizing the cell membrane. Their release leads to destabilization of the bacterial membrane. Additionally, some authors suggest that this release of divalent cations further stimulates bacterial autolysins, contributing to bacterial cell lysis.

In addition to its antibacterial properties, lysozyme has demonstrated a fungicidal effect, particularly against *Candida albicans*. The precise mechanism underlying this fungicidal action remains subject to debate. Two possible mechanisms have been proposed:

- The first involves enzymatic hydrolysis of N-glucosidic bonds connecting polysaccharides and structural proteins in fungal cells, leading to damage of the fungal cytoplasmic membrane.
- The second mechanism involves damage to the *Candida albicans*

plasmolemma, which is followed by activation of mannan synthase, chitin synthase, and potentially other enzymes crucial for membrane stability.

Lysozyme present in saliva constitutes a key component of nonspecific antimicrobial defense in the oral cavity. Multiple studies have demonstrated a negative correlation between salivary lysozyme concentration and dental biofilm accumulation as well as gingivitis occurrence in children and young adults. Additionally, increased salivary lysozyme levels have been observed in patients with oral candidiasis.

In addition to its antibacterial and fungicidal activities within the oral cavity, salivary lysozyme inhibits the adherence of *Streptococcus mutans* and *Streptococcus sanguis* to the acquired dental pellicle. This action reduces bacterial accumulation and consequently diminishes dental biofilm formation.

Salivary lysozyme tends to form complexes, known as salivary micelles, with other salivary proteins including mucin, lactoferrin, amylase, secretory IgA (sIgA), and proline-rich proteins. These protein-protein interactions are mediated through various chemical bonds, such as ionic, hydrogen, and hydrophobic bonds. The formation of these salivary micelles enhances bacterial aggregation within the oral cavity, thereby contributing to the regulation of the oral microbiota.

LACTOFERRIN

Lactoferrin is a glycoprotein by structural classification. It belongs to the transferrin family, comprising non-heme iron-binding proteins. Besides lactoferrin, the transferrin family includes transferrin, the primary iron transport protein in blood plasma, ovotransferrin, found in chicken egg whites, melanotransferrin, a protein expressed by melanocytes, and the recently identified carbonic anhydrase inhibitor.

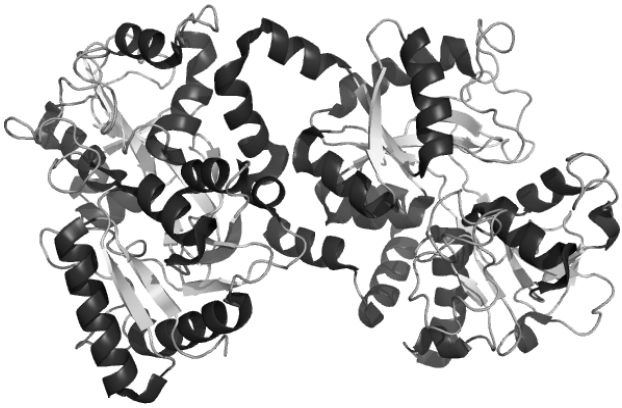


Figure 4.2: *Three-dimensional structure of lactoferrin*

Lactoferrin is a monomeric protein comprising 692 amino acids (Figure 4.2). The protein consists of a single polypeptide chain, which is divided into two domains: the amino-terminal domain (N-terminal), containing up to 333 amino acid residues, and the carboxy-terminal domain (C-terminal), spanning residues 345 to 692. These two domains are connected by a triple alpha-helix composed of approximately a dozen amino acids (residues 334 to 344). Lactoferrin has a molecular mass of approximately 80 kDa and is a highly cationic molecule with an isoelectric point (pI) ranging from 8.4 to 9. Its positive charge enables binding to anionic molecules, and it has also been demonstrated to interact with various cell types through negatively charged phospholipid groups in their membranes. Nonetheless, lactoferrin's primary function is as an iron-binding protein, playing a critical role in the homeostasis of this essential bioelement.

Each Fe^{3+} ion is coordinated by specific amino acid residues within the lactoferrin molecule—namely Tyr92, Tyr192, Asp60, and His253—in the presence of bicarbonate ions, which stabilize the interaction through electrostatic charge balance. By sequestering iron in this manner, lactoferrin prevents oxidative modifications of this bioelement's valency thereby exerting a significant antioxidant effect. In contrast, unbound (free) iron can catalyze the formation of reactive oxygen species during oxidation reactions within cells, leading to oxidative damage of biomolecules such as proteins, lipids, and DNA.

The primary source of lactoferrin is milk, from which the protein derives its name. The highest concentration is found in human colostrum, reaching approximately 7 g/L. Lactoferrin constitutes a key component of the body's innate antimicrobial defense mechanisms, serving as the first line of protection of mucosal surfaces against pathogenic microorganisms.

In the oral cavity, lactoferrin is present in saliva and originates from both the epithelial cells of the oral mucosa and infiltrating leukocytes. Notably, there are significant variations in lactoferrin concentration across different types of salivary secretions (Table 4.2).

Table 4.2: Lactoferrin concentration in different types of salivary secretion

Types of salivary secretion	Lactoferrin concentration (g/ml)
Parotid salivary gland	
stimulated	4,7 – 12
unstimulated	6,8 – 20
Submandibular and sublingual saliva	
stimulated	11
unstimulated	13
Mixed saliva	
stimulated	8,5 – 24

Lactoferrin has antibacterial, antiviral, and fungicidal effects.

Antibacterial effect of lactoferrin

Lactoferrin exerts both bacteriostatic and bactericidal effects against a wide range of Gram-positive and Gram-negative bacteria. Its primary biological function is the high-affinity binding of iron, through which it contributes to the regulation of iron homeostasis in body fluids. By sequestering iron, lactoferrin renders it unavailable to bacteria, depriving them of an essential element required for their metabolic activity. This mechanism, referred to as **"nutritional immunity,"** effectively inhibits bacterial growth and proliferation.

The significance of lactoferrin's iron-binding capacity is particularly evident in patients with aggressive periodontitis. Specifically, studies have shown that salivary lactoferrin in these individuals exhibits a diminished ability to sequester iron. As a result, this bioelement remains available to support the growth and proliferation of *Aggregatibacter actinomycetemcomitans*, a virulent microorganism responsible for severe destruction of periodontal tissues.

The bactericidal activity of lactoferrin involves its direct interaction with the bacterial membrane. As a cationic molecule, lactoferrin binds to

lipopolysaccharides (LPS) present in the bacterial membrane. This interaction facilitates the release of LPS from the membrane. Consequently, lactoferrin compromises the structural integrity of the bacterial cell membrane.

In addition to exerting a bacteriostatic effect against numerous pathogenic bacteria, lactoferrin also has the capacity to promote the growth of certain bacterial species. Its probiotic effect has been demonstrated, specifically through stimulation of the growth of probiotic strains such as *Lactobacillus acidophilus* and *Bifidobacterium* spp.

Antiviral effect of lactoferrin

Lactoferrin also exhibits antiviral activity, primarily through its ability to inhibit the replication of a wide range of viruses under *in vitro* conditions. However, numerous studies have demonstrated that lactoferrin primarily prevents viral invasion of host cells, while its effect on inhibiting viral replication is comparatively limited.

Lactoferrin impedes the entry of viruses into host cells by the following two mechanisms:

- through direct binding of lactoferrin to the virus (e.g., hepatitis C virus, poliovirus, rotavirus, herpes simplex virus, and human immunodeficiency virus – HIV);

- through binding of lactoferrin to host cells, specifically to biomolecules within the cell membrane that serve as viral receptors or coreceptors. For example, lactoferrin can bind to heparan sulfate proteoglycans (Figure 4.3) on host cells. Many viruses are capable of attaching to these membrane components during the initial phase of infection. Upon this first interaction, the virus typically gains entry into the cell. However, by occupying these receptor sites, lactoferrin blocks viral attachment and thereby prevents viral entry into the host cell.

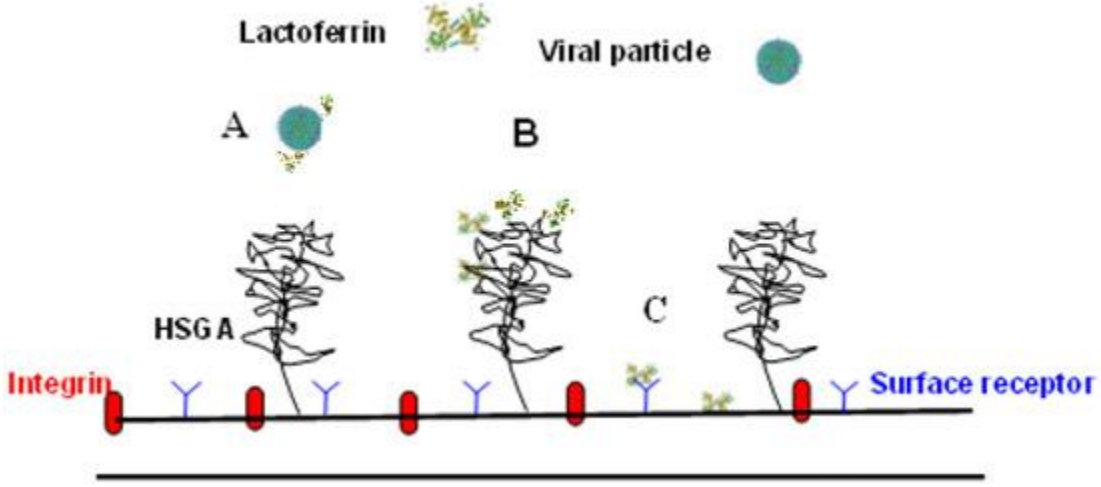


Figure 4.3: *Different mechanisms of prevention of viral infection by lactoferrin. A – Direct binding of lactoferrin to the virus. B, C – Binding of lactoferrin to receptors on host cells.*

Fungicidal effect of lactoferrin

Lactoferrin exerts a similar effect on the fungus *Candida albicans* as it does on bacteria. This primarily involves its ability to bind iron, as well as its direct interaction with *Candida albicans*, leading to disruption of fungal membrane permeability.

HISTATINS

Histatins are a family of low molecular weight salivary proteins characterized by a high content of the amino acid histidine, which is why they are also referred to in the literature as histidine-rich proteins. Their primary source is the major salivary glands—parotid, submandibular, and sublingual—placing them in the category of salivary proteins of local origin. The most notable members of the histatin family include histatin 1, histatin 3, and histatin 5. Histatins 3 and 5 are highly basic peptides, with an isoelectric point (pI) greater than 9.5, whereas histatin 1 is neutral, with a pI of 7.

A distinguishing characteristic of histatins is their post-translational modification, particularly observed in histatins 1 and 3. These modifications include phosphorylation of the second serine residue in the peptide chain of histatin 1 and sulfation of a tyrosine residue within the same peptide. Additionally, due to the proteolytic activity present in saliva, numerous proteolytic fragments of histatins are found in this biological fluid. As a result of

both post-translational modifications and salivary proteolysis, in addition to the primary representatives (histatins 1, 3, and 5), saliva also contains proteolytic fragments such as histatins 2, 4, 6, 7, 8, 9, 10, 11, and 12. A considerable number of small peptides have been identified in saliva that exhibit biological activity homologous to that of histatins.

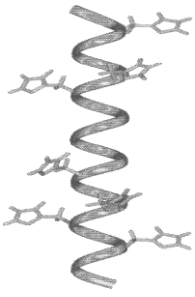
Histatins, based on their chemical structure, are classified as peptides enriched with basic amino acids such as histidine (7 residues), arginine, and lysine. Consequently, histatins exhibit a basic character and demonstrate cathodic electrophoretic mobility. Histatin 1 comprises 38 amino acids and has a molecular mass of 4929 Da. Histatin 3 contains 32 amino acids with a molecular mass of 4063 Da, while histatin 5 consists of 24 amino acids and a molecular mass of 3037 Da (Table 4.3).

Table 4.3: Biochemical characteristics of histatins

	Number of amino acids	Molecular mass (Da)	pI
Histatin 1	38	4929	>9,5
Histatin 3	32	4063	>9,5
Histatin 5	24	3037	7

There are both structural differences and similarities among the major salivary histatins (1, 3, and 5). Histatin 1 contains a phosphorylated serine residue, whereas histatins 3 and 5 possess serine residues without phosphorylation. A common feature of all three histatins is the presence of seven histidine residues. Additionally, the amino-terminal region, consisting of 22 amino acids, is identical in all three histatins. In histatins 1 and 3, seven identical amino acids are also found in the carboxy-terminal region. The entire sequence of histatin 5 corresponds to the N-terminal portion of histatin 3. These findings suggest that histatins 1 and 3 are products of two distinct structural genes, while histatin 5 is a proteolytic derivative of histatin 3 (Figure 4.4).

Figure 4.4: *Structure of histatin*



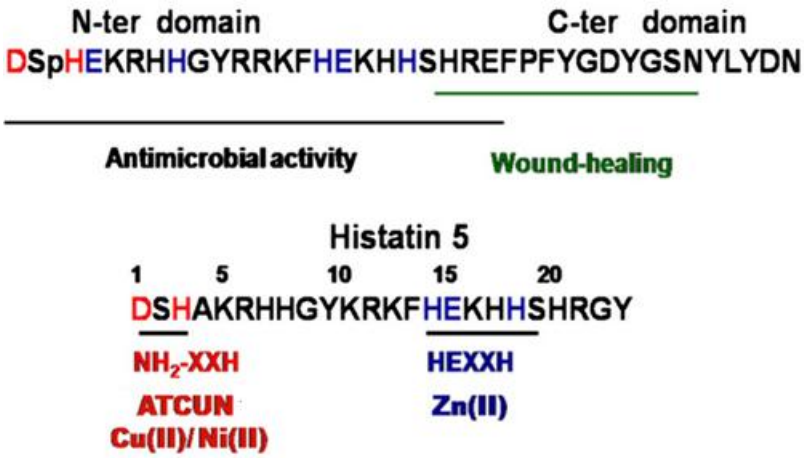


Figure 4.5: *Sequences of histatin 1 and histatin 5 with their functional domains and domains with which they bind certain metals. Figure 4.5 shows the sequences of histatin 1 and histatin 5 with their functional domains. Two sequences are observed in the N-terminal domain of histatin 5. One is the so-called ATCUN (amino-terminal Cu(II)/Ni(II)) sequence, which contains a single histidine residue at the third position and binds Cu(II) and Ni(II), present in saliva. The second sequence is a zinc-binding (HEXXH) sequence and is a characteristic sequence for several different metalloproteinases. Histatin 5 binds Zn(II) and Cu(II) even at a very low dissociation constant. This indicates that these metals in saliva bind very easily to histatin 5. The calcium present in saliva does not interfere with the binding of Zn(II) and Cu(II) to the molecule of this peptide. This indicates the low affinity for binding of Ca ion and histatin 5.*

The concentration of histatins in saliva is relatively low compared to the total salivary protein content. Histatins constitute approximately 2.6% of the total protein content in parotid and submandibular/sublingual saliva. In parotid saliva, histatin concentration ranges from 30 to 425 µg/mL, whereas in submandibular/sublingual secretion, their concentration ranges from 55 to 347 µg/mL. In unstimulated saliva, histatin levels are significantly higher in parotid saliva than in submandibular/sublingual saliva. Upon stimulation of salivary secretion, histatin concentration decreases in parotid saliva but increases in submandibular/sublingual saliva. This variation in histatin concentration is attributable to changes in total protein concentration, resulting from alterations in saliva volume secreted. In submandibular/sublingual gland secretion, the total protein concentration increases upon stimulation, contrasting with parotid saliva, where total protein concentration decreases with stimulation. Notably, advancing age does not affect the concentration of total salivary proteins; however, histatin

concentration declines with age. In parotid saliva, histatin levels peak around midday, whereas in submandibular/sublingual saliva, the highest concentration is observed in the morning hours.

Histatins are crucial for various biological processes within the oral cavity, contributing to the maintenance of oral homeostasis and the preservation of oral tissue health. In the oral environment, histatins fulfill the following functions:

- Formation of the acquired dental pellicle on tooth surfaces;
- Non-specific humoral antibacterial and fungicidal protection;
- Binding to specific metal ions present in saliva;
- Inhibition of selected proteolytic enzymes and inflammatory mediators;
- Binding of toxic polyphenols within the oral milieu;
- Promotion of wound healing.

Formation of dental pellicle

Dental pellicle is an acquired organic film that develops on the surface of tooth enamel. Its formation is especially pronounced during mastication. Biochemically, the dental pellicle consists of various salivary proteins and glycoproteins. This pellicle is formed through the adsorption of these salivary components onto the enamel surface.

Histatin 1 plays a pivotal role in the formation of this conditionally beneficial pellicle. It selectively adsorbs to the hydroxyapatite crystals present in the tooth enamel.

Antibacterial and fungicidal properties of histatins

Histatins belong to the group of cationic peptides exhibiting antimicrobial properties. Given that the oral cavity serves as the primary “entry point” for numerous microorganisms from the external environment, the presence of abundant salivary biomolecules with defensive and antimicrobial functions is critically important. Histatins fulfill this essential protective role within the oral environment.

Histatins exhibit activity against specific bacteria implicated in dental caries (Streptococcus mutans) and periodontitis (Fusobacterium nucleatum, Prevotella intermedia). Notably, histatin 5 demonstrates significant antimicrobial activity against the periodontal pathogen Porphyromonas gingivalis. Owing to their positive charge, these cationic peptides engage in electrostatic interactions with the negatively charged bacterial cell wall. This electrostatic binding and subsequent accumulation of histatins within the lipid bilayer of the bacterial membrane result in membrane thinning and bacterial lysis. Antibacterial effects are also observed from proteolytic fragments of histatins, as well as salivary peptides possessing biological activity analogous to that of histatins.

Histatin 5 exhibits both fungistatic and fungicidal activity in "*in vitro*" conditions against a broad spectrum of fungi, including *Candida albicans*, *Candida glabrata*, *Candida krusei*, *Saccharomyces cerevisiae*, and *Cryptococcus neoformans*.

Oral fungal infections are often associated with mechanical trauma to the oral mucosa, disruption of the normal oral microbiota, and immunodeficiency conditions such as HIV infection. Numerous studies have investigated the fungicidal properties of histatins, particularly histatin 5; however, its precise mechanism of action remains incompletely understood. It is established that histatin 5 does not function as a classical antibiotic by forming pores or ion channels in the *Candida albicans* membrane that would lead to membrane destabilization and disintegration.

Research has demonstrated that the action of histatin 5 on *Candida albicans* involves several distinct phases:

- binding to specific receptors on the fungal membrane;
- transport across the membrane into the cytoplasm;
- intracellular translocation; and
- efflux of K^+, Mg^{2+}, and ATP ions from the cell.

The receptor on the *Candida albicans* membrane to which histatin 5 binds is a protein with an approximate molecular mass of 70 kDa. This protein belongs to the family of "heat-shock" proteins. The intracellular target of histatin 5 in *Candida albicans* is the mitochondrion, where this salivary protein inhibits the respiratory chain. Fluorimetric analyses have shown that histatin 5 promotes the generation of oxygen free radicals within mitochondria. These reactive oxygen species induce oxidative degradation of the mitochondrial membrane,

damage biomolecules, disrupt cellular metabolism, and ultimately lead to cell death (Figure 4.6).

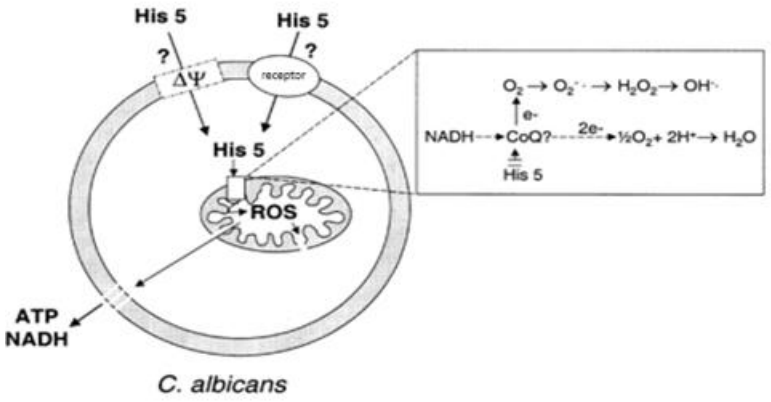


Figure 4.6: *Proposed mechanism of fungicidal action of histatin*

Due to this fungicidal effect and the non-toxicity of histatins to human tissues, their potential use as antifungal agents in the treatment of oral candidiasis is currently under investigation.

Binding to metal ions

Histatins possess the capacity to bind specific metal ions present in saliva. Within the N-terminal domain of histatin 5, two distinct sequences are identified. The first is the ATCUN (amino-terminal Cu(II)/Ni(II)) sequence, containing a histidine residue at the third position, which binds Cu(II) and Ni(II) ions found in saliva. The second is the zinc-binding (HEXXH) motif, characteristic of several metalloproteinases. The interaction with these metal ions is crucial for protecting tooth enamel and regulating the growth and proliferation of oral microorganisms. Furthermore, binding of zinc and copper enhances histatins' oxidative antimicrobial activity against bacteria and Candida albicans.

Inhibition of bacterial and tissue proteolytic enzymes and mediators implicated in the pathogenesis of periodontal disease

Periodontal disease is a chronic infectious inflammatory condition characterized by destruction of periodontal fibers, resorption of alveolar bone, and formation of periodontal pockets. Numerous studies have demonstrated that during gingival inflammation and periodontitis, there is increased release of proteolytic enzymes and inflammatory mediators, originating either from bacteria or from defense cells within periodontal tissues. Besides bacterial proteinases, matrix metalloproteinases (MMPs) constitute another critical group of enzymes involved in the pathogenesis of periodontal disease. MMPs are secreted by host defense cells and are considered primary mediators of extracellular matrix protein degradation. These enzymes belong to the metalloenzyme family, specifically zinc-dependent proteases. Matrix metalloproteinases are capable of degrading extracellular matrix proteins such as collagen, elastin, fibronectin, and laminin. These enzymes also play a vital role in the physiological processes of extracellular matrix turnover, being integral to development, morphogenesis, and tissue remodeling. However, beyond their involvement in normal physiological functions, dysregulated matrix metalloproteinase activity contributes significantly to various pathological conditions, including arthritis, tumor metastasis, and periodontal diseases. Notably, saliva from patients with periodontal disease exhibits markedly increased activity of MMP-2 and MMP-9 compared to individuals without periodontitis. Inhibition of matrix metalloproteinases represents a potentially significant approach for the treatment of periodontal diseases. These enzymes are zinc-dependent, with zinc constituting a crucial component of their active site; therefore, sequestration of zinc results in their inactivation. As previously noted, histatin 5 forms complexes with metal cations, particularly zinc. Through this mechanism, histatin 5 acts as an inhibitor of enzymes that require zinc for catalytic activity, including matrix metalloproteinases such as MMP-2 and MMP-9.

During the pathogenetic progression of periodontal disease, tissue destruction results from the increased release and heightened activity of enzymes secreted both by host defense cells and by bacteria. Porphyromonas gingivalis and Clostridium histolyticum are anaerobic, gram-negative bacteria residing in the subgingival biofilm, playing a key role in the etiology of periodontal diseases. The proteinases released by these microorganisms degrade several

physiologically important proteins, including collagen, fibrin, fibrinogen, fibronectin, immunoglobulins, and complement proteins. Histatin 5 has the capacity to inhibit some of these bacterial proteinases, such as trypsin-like enzymes, cysteine proteinases, and collagenases.

Tissue destruction during periodontitis is also mediated by various signaling molecules released from defense cells in response to bacterial infection. These molecules, known as cytokines, play a key role in the inflammatory process. The outer membrane of Porphyromonas gingivalis contains a protein that stimulates the release of inflammatory mediators such as interleukin 6 (IL-6) and interleukin 8 (IL-8) from polymorphonuclear leukocytes and periodontal tissue fibroblasts. Experimental evidence has demonstrated that histatin 5 inhibits the release of these inflammatory mediators by fibroblasts.

The bacterium Aggregatibacter actinomycetemcomitans is a facultative anaerobic, gram-negative microorganism that plays a significant role in the etiology of periodontitis, particularly in aggressive forms of the disease. Its pathogenicity is primarily attributed to its ability to destroy polymorphonuclear cells, monocytes, and T-lymphocytes. This destructive effect is mediated by a bacterial protein called leukotoxin, which has a molecular weight of approximately 115 kDa. Studies have demonstrated that histatin 5 can inhibit leukotoxin; however, the precise mechanism underlying this protective effect remains to be elucidated.

Binding of polyphenols within the oral milieu

Histatins possess the ability to bind toxic polyphenols, thereby playing a significant role in the detoxification of dietary tannins. Dietary tannins are polyphenolic compounds known to exert harmful effects on the body, particularly impacting the lungs and liver. Since these tannins are ingested through plant-based foods, the oral cavity—with its salivary proteins—serves as the first line of defense against such harmful agents. Proline-rich proteins (PRPs) primarily mediate the formation of complexes with tannins. By binding tannins, PRPs prevent their interaction with other biomolecules. Due to their hydrophobic nature and high proline content, PRPs exhibit a strong affinity for tannins. Recent evidence indicates that histatins also bind polyphenol derivatives. Unlike PRPs, histatins have a low molecular mass and lack proline residues. Consequently, the binding of tannins by histatins is primarily facilitated by the basic and aromatic amino acids within their structure. Through the formation of insoluble tannin–histatin complexes, which are not absorbed in the digestive tract, histatins

effectively neutralize the harmful effects of tannins.

Promotion of wound healing

Recently, another significant function of histatins has been identified, related to the maintenance of soft tissue integrity in the oral cavity. In cases of oral wounds, the concentration of histatins in saliva exceeds that of epidermal growth factor (EGF). The oral mucosa is frequently subjected to mechanical forces that result in tissue injury. Saliva contributes to the repair of these injuries through multiple mechanisms. Several growth factors present in saliva have been identified as participants in the healing process of oral mucosal wounds, including EGF, nerve growth factor, fibroblast growth factor, and trefoil factor 3. Epidermal growth factor (EGF) plays a critical role in several cellular processes essential for wound healing, including cell differentiation, proliferation, and migration. Both EGF and rodent nerve growth factor have been demonstrated to be key contributors to wound repair. However, the concentrations of these two growth factors in human saliva are approximately 100,000 times lower than those found in rodent saliva.

These data indicate that EGF is not the key determinant in wound healing. Recently, it has been discovered that the C-terminal domain of histatins 1 and 3 contains a sequence responsible for promoting re-epithelialization and wound repair. Wound healing occurs in several phases: inflammation, proliferation, and maturation. Epithelial cell migration and proliferation are critical factors for re-epithelialization and angiogenesis. Histatin 1 stimulates epithelial cell migration and proliferation when its concentration in saliva ranges from 1 to 10 μM. Activation of cell migration by histatin and EGF is regulated through distinct intracellular mechanisms. Histatins activate G-proteins located in the cell membrane, thereby initiating cellular processes that promote proliferation and migration (Figure 4.7).

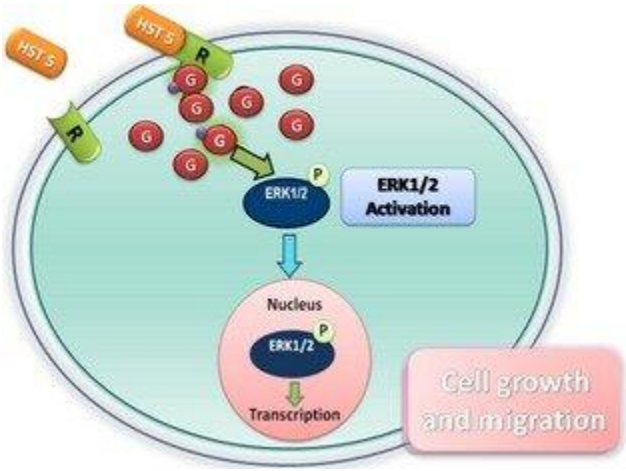


Figure 4.7: *Schematic representation of the mechanism of histatins in wound healing. Histatins interact with an unknown receptor on the cell membrane and activate the G-protein. This is followed by intracellular activation of various kinases and phosphorylation, and as a result, cell growth and migration are stimulated.*

DEFENSINS

Defensins belong to the group of salivary proteins with antimicrobial properties, which also includes peroxidase, lactoferrin, lysozyme, histatins, and calprotectin. They are small, low-molecular-weight proteins (3–5 kDa), rich in cysteine residues and exhibit cationic electrophoretic mobility (Table 4.4). Defensins possess antimicrobial activity against various gram-positive and gram-negative bacteria, fungi such as Candida albicans, and certain viruses including Herpes simplex. Their primary bactericidal mechanism involves the formation of micropores or channels in the bacterial cell membrane. Defensins are categorized into two main groups: α-defensins and β-defensins.

Table 4.4: Biochemical characteristics of defensins

Type of defensis	Molecular mass (Da)	Disulfide bonds
HND-1	3442	2-30, 4-19, 9-29
HND-2	3371	9-29
HND-3	3486	1-29, 3-18, 8-28
HND-4	3709	2-30, 4-19, 9-29
HND-5	3938	2-30, 4-19, 9-29
HND-6	4053	6-34, 8-23
HBD-1	3929	13-33, 5-34, 12-27, 17-35
HBD-2	4328	8-37, 15-30, 20-38

α-Defensins (classical defensins) are short peptides composed of 29–35 amino acids. Six α-defensins have been identified in human tissues (Figure 4.8). Four of these α-defensins are found in neutrophil granulocytes and are referred to as human neutrophil defensins (HND-1 to HND-4). The remaining two α-defensins (HND-5 and HND-6) have been isolated from Paneth cells of the small intestine and epithelial cells of the female urogenital tract.

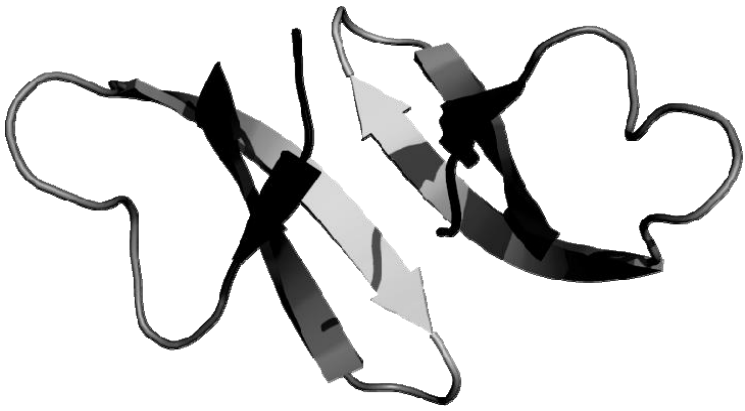


Figure 4.8: *Structure of α-defensins*

Following their synthesis, these proteins undergo post-translational modifications. Specifically, from the preprodefensins (comprising 93 to 94 amino acids), the N-terminal peptide is cleaved by proteolysis, yielding the mature defensin represented by the C-terminal peptide. This mature defensin contains three intramolecular disulfide bonds and exhibits cationic electrophoretic mobility.

HND-1, HND-2, and HND-3 have been identified in the mixed saliva of healthy individuals. The concentration of HND-1 in saliva averages 8.6 ± 8 μg/ml, while HND-2 is present at approximately 5.6 ± 5.2 μg/ml. During inflammatory conditions within the oral cavity, the salivary concentration of α-defensins increases significantly.

β-Defensins are short peptides composed of 41 amino acids (Figure 4.9). Their structure is stabilized by three intramolecular disulfide bonds. Six types of human β-defensins (HBD-1 through HBD-6) have been identified. These peptides are produced by epithelial cells of various organs, including the skin, lungs, kidneys, pancreas, uterus, and eyes. Expression of three β-defensins—HBD-1, HBD-2, and HBD-3—has been documented in epithelial cells of the oral mucosa (keratinocytes) and in the ductal cells of salivary glands.

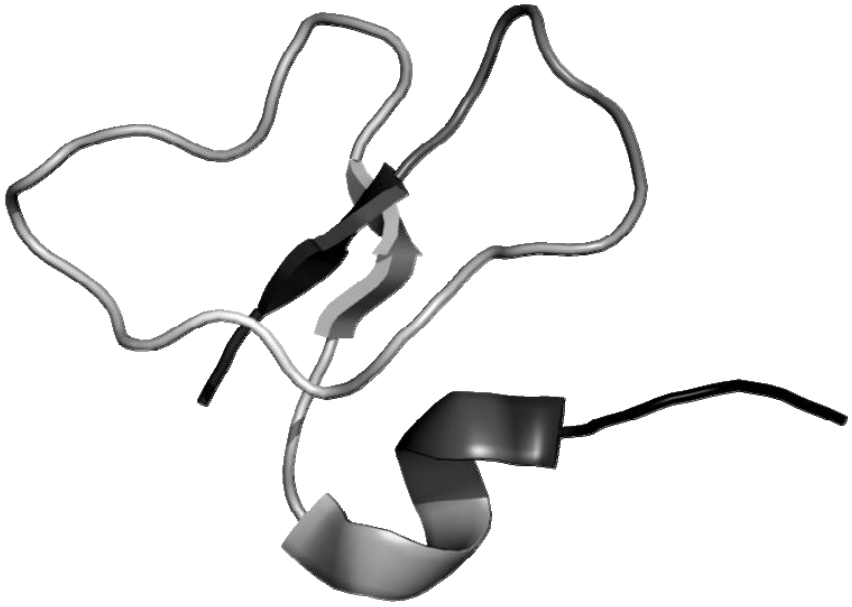


Figure 4.9: *Structure of β-defensins*

β-Defensins, besides being present in saliva, have also been identified in other bodily secretions such as urine, bronchial fluid, and nasal secretions. The concentration of HBD-1 and HBD-2 in saliva is approximately 150 μg/ml. Their primary source in saliva is the keratinocytes of the oral mucosa, with a lesser contribution from the ductal epithelial cells of the salivary glands.

The antimicrobial activity spectrum of defensins is extensive, targeting a wide array of gram-positive and gram-negative bacteria, fungi, and certain viruses, including HIV. Defensins play a crucial role in maintaining oral health by contributing to local defense mechanisms that prevent microbial colonization on the oral mucosa. Alongside other antimicrobial peptides, defensins form an essential part of the first line of defense of the oral mucosa against diverse infectious agents.

In saliva, defensins can be masked through the formation of complexes with other salivary proteins. Salivary mucins, which are large anionic molecules, are known to form complexes with IgA, lysozyme, histatins, and proline-rich proteins (PRPs). Mucins are large anionic molecules that can also bind small cationic molecules such as defensins (e.g., HBD-1). These complexes protect defensins from degradation by bacterial proteases and may enhance their function by facilitating their attachment to the oral mucosa.

The mechanism of the antimicrobial action of defensins can be divided into several stages:

1. Electrostatic binding of defensins, which are cationic peptides, to the bacterial cell membrane that carries an overall anionic charge.
2. Increase in bacterial membrane permeability. This occurs via two mechanisms: first, by forming ion channels; second, through the "carpet model," which implies aggregation of these peptides and creation of a transit path for their passage.
3. Disruption of protein synthesis within the bacterial cell

Due to their potent antimicrobial activity, defensins are often referred to as “natural antibiotics.” Some studies have explored their potential use in treating oral diseases, particularly periodontitis. However, their clinical application is limited by the cytotoxic effects of β-defensins at high concentrations.

CALPROTECTIN

Calprotectin is an antimicrobial protein structurally characterized as a heterodimer composed of two peptide chains: a light chain known as MRP 8 and a heavy chain referred to as MRP 14. The molecular mass of calprotectin is approximately 24 kDa. Upon binding calcium ions, calprotectin undergoes conformational changes at its calcium-binding sites, which facilitates the association of additional peptide chains with the molecule. Furthermore, calprotectin contains specific binding sites for zinc ions.

Calprotectin was initially isolated from neutrophil leukocytes and mononuclear phagocytes. In neutrophil leukocytes, calprotectin constitutes approximately 30–60% of the total cytoplasmic proteins of these immune cells. This protein is secreted into the extracellular space by activated neutrophils and monocytes or released following the disruption of the cellular integrity of these cells. Immunohistochemical studies have further confirmed the presence of calprotectin within the membranes of non-keratinized epithelial cells. Calprotectin expression has also been demonstrated in the cytoplasm of certain mucosal epithelial cells. Its soluble form has been detected in blood plasma, urine, and various other bodily secretions. In saliva, calprotectin has been identified primarily originating from gingival fluid, mucosal transudate, and gingival keratinocytes.

Calprotectin possesses antimicrobial properties and has the ability to induce apoptosis, which is a form of controlled programmed cell death. By binding zinc ions, calprotectin inhibits the activity of matrix metalloproteinases, a family of zinc-dependent enzymes that play crucial roles in embryonic development, angiogenesis, wound healing, inflammation, carcinogenesis, and tissue destruction. Through this mechanism, calprotectin contributes to the regulation of numerous vital physiological processes. Additionally, calprotectin suppresses microbial growth by competing for zinc. The binding of zinc to the histidine-rich regions of calprotectin represents a crucial mechanism of nonspecific antimicrobial host defense. Microorganisms require essential bioelements, such as zinc, for their metabolism and reproduction. However, due to calprotectin's high zinc-binding capacity, it effectively deprives microorganisms of this vital element, thereby inhibiting their survival and proliferation. Calprotectin, at concentrations ranging from 50 to 250 μg/ml, can inhibit the growth of bacteria including *Escherichia coli*, *Staphylococcus aureus*, and *Staphylococcus epidermidis*. At lower concentrations, between 4 and 32 μg/ml, calprotectin is also capable of inhibiting the growth of the fungus *Candida*

albicans.

In addition to its role in nonspecific antimicrobial protection, calprotectin fulfills two other important biological functions. These are the following:

- it regulates inflammatory processes at the cellular level
- it exerts a cytotoxic effect by inhibiting the growth and proliferation of a wide range of tumor cells.

CATHELICIDINS

Cathelicidins are small peptides characterized by cationic electrophoretic mobility and comprising between 12 and 100 amino acids, exhibiting antimicrobial activity. These peptides are initially synthesized as preproteins composing an N-terminal signal peptide (up to 30 amino acids), a cathelicidin prosequence (94–144 amino acids), and a C-terminal peptide (12–100 amino acids). In human tissues, the proform of cathelicidins exists as an 18 kDa cationic antimicrobial peptide known as hCAP-18, which is predominantly localized within the specific granules of neutrophil leukocytes. Upon neutrophil degranulation, proteolytic cleavage of the C-terminal peptide from hCAP-18 by proteinase-3 generates the biologically active form of cathelicidins, designated LL-37.

Cathelicidins are distributed throughout the skin and mucous membranes and are also present in saliva and sweat. Within the oral cavity, cathelicidins have been identified in tissues such as the tongue, buccal mucosa, gingiva, and salivary glands. The presence of cathelicidins in saliva, alongside other antimicrobial peptides, constitutes a crucial component of the nonspecific immune defense of the oral environment. In the oral cavity, cathelicidins contribute to the following biological functions:

- The primary function of the human peptide LL-37 is its antimicrobial activity, which targets a broad spectrum of gram-positive and gram-negative bacteria, fungi, viruses, and parasites. The neutralization of bacteria by LL-37 occurs rapidly and is mediated through the formation of ion channels or pores in the bacterial membrane. Cathelicidins have been shown to bind to lipopolysaccharides (LPS) on the bacterial membrane, thereby compromising the integrity of the bacterial cell. Specifically, cathelicidins, similar to other cationic antimicrobial peptides, interact with the binding sites on LPS that normally coordinate divalent cations. These peptides exhibit a higher affinity for LPS than some

divalent cations such as Ca^{2+} and Mg^{2+}. Thus, aforementioned divalent ions are displaced by the cathelicidins subsequently disrupting the membrane permeability.

- Cathelicidins also play a significant role in tissue healing processes.
- They inhibit tissue damage caused by the harmful effects of free radicals. These free radicals are generated by phagocytes as a result of the tissue's response to bacterial infection. While these toxic oxidants are effective in killing bacteria, they can also damage host tissues through oxidative mechanisms. For this reason, it is very important that the release of free radicals from phagocytes is precisely regulated. One of the ways to achieve this regulation is by modifying the activity of the enzyme NADPH oxidase, which participates in the production of free radicals by phagocytes. Cathelicidins are capable of inhibiting the mentioned enzyme, thereby reducing the production of free radicals by phagocytes.

ADRENOMEDULLIN

Adrenomedullin is a cationic, multifunctional antimicrobial peptide composed of 52 amino acids. It is found in gingival fluid, saliva produced by the salivary gland parenchyma, and whole saliva. It is also presumed that epithelial cells of the oral mucosa contribute to its production. Due to its positive charge, adrenomedullin exhibits bactericidal activity by binding to bacterial membranes and disrupting their integrity. This disruption occurs through the formation of ion channels or transmembrane pores within the bacterial membrane. Additionally, this small cationic peptide inhibits the proliferation and growth of certain bacterial species, such as *Staphylococcus aureus*, by interfering with bacterial cell division.

BACTERICIDAL/PERMEABILITY INCREASING PROTEIN

These are lipid-binding proteins that share similar molecular characteristics. Bactericidal/permeability-increasing proteins (BPI) are cationic and have a molecular mass of approximately 52 kDa. Their primary sources include neutrophil granulocytes, epithelial cells of the oral mucosa, and the parotid salivary glands. These salivary proteins exhibit key functional properties: a bactericidal effect, neutralization of bacterial endotoxins, and opsonizing activity. The bactericidal activity and the neutralization of lipopolysaccharides (bacterial endotoxins) are mediated by the N-terminal domain of the molecule, whereas the opsonizing function is attributed to the C-terminal domain.

5 OTHER SALIVA PROTEINS INVOLVED IN NON-SPECIFIC IMMUNOLOGICAL DEFENSE OF THE ORAL CAVITY

MUCINS (sialomucins)

- **Coating role of mucin**
- **Antimicrobial effect of mucin**

SALIVARY AMYLASE (SELF-CLEANING OF THE ORAL CAVITY)

- **Biochemical characteristics of salivary amylase**

PROLINE RICH PROTEINS (PRPs)

- **Protection of tooth enamel**
- **Binding of dietary tannins**
- **Antimicrobial effect**
- **Formation of the acquired dental pellicle**

LINGUAL LIPASE

SALIVARY CARBONIC ANHYDRASE (CA)

ORAL PEROXIDASE

CYSTATINS

STATHERINS

MUCINS (sialomucins)

Twenty distinct classes of mucins have been identified in the human body. These glycoproteins are expressed on the surface of epithelial cells lining the gastrointestinal, respiratory, and genitourinary tracts, as well as the ocular surface (Figure 5.1). Each class of mucin possesses a specific structural configuration that correlates with its anatomical localization and corresponding function.

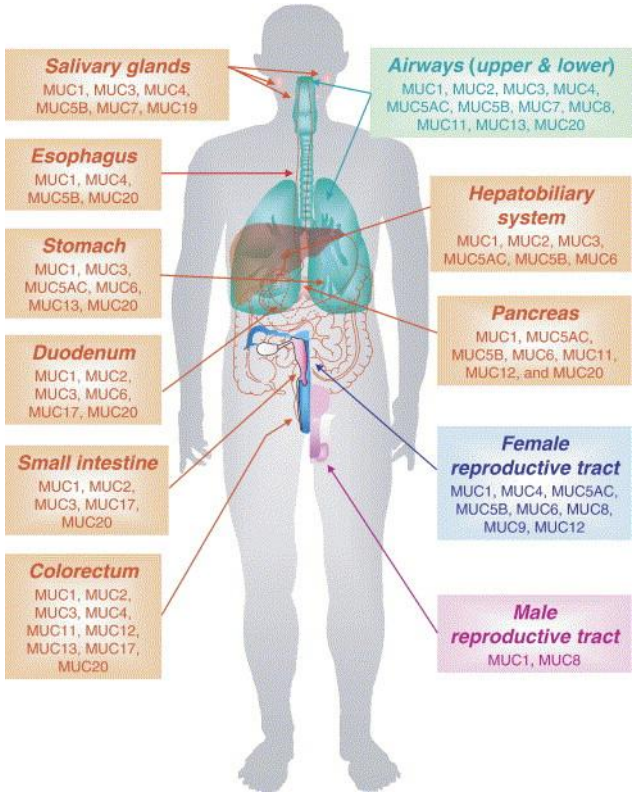


Figure 5.1: *Mucin in the human body*

The following classes of mucins have been identified in the oral cavity: MUC5B (also referred to as MG1), MUC7 (or MG2), MUC19, MUC1, and MUC4. Although salivary mucins collectively contribute to the maintenance of oral tissue integrity, each mucin class exhibits distinct biochemical structures and functional properties, which vary according to their specific localization within the oral cavity.

MG1 plays a predominant role in the formation of the mucous gel layer within the oral cavity and is secreted by mucous cells of the submandibular, sublingual, palatine, and labial salivary glands. Although MUC19 also contributes to mucous gel formation, MG1 remains the principal mucin associated with this function in the oral environment.

MG2 is present in saliva in monomeric or dimeric forms and does not participate in the formation of the mucous gel layer. However, monomers and dimers of this mucin class are capable of associating to form larger complexes, thereby contributing to the aggregation of bacterial cells in the oral cavity. The expression of this low-molecular-weight mucin in the acinar cells of the salivary glands exhibits interindividual variability. Its presence has been confirmed in the mucous acini of the submandibular and sublingual glands, while its occurrence in the serous cells of these glands appears to be inconsistent.

MUC1 and MUC4 are mucins that are present on the membranes of the duct cells of the parotid and submandibular glands, as well as on the membranes of the duct cells of the small mucous glands. They are thought to have a function in transducing the cellular signal for salivary secretion.

Mucins (MG) are high-molecular-weight glycoproteins composed of a protein core and a carbohydrate component, with the latter comprising more than 50% of the molecule. The predominance of mucins in saliva contributes to its characteristic viscosity. Mucins have molecular weights ranging from 100,000 to several million Daltons and are primarily secreted by the submandibular and sublingual glands. This section focuses on the two most extensively studied classes of salivary mucins: MUC5B (type 1 – MG1) and MUC7 (type 2 – MG2). MUC5B (MG1) is distinguished by its very high molecular weight ($>10^6$ Da), whereas MUC7 (MG2) exhibits a considerably lower molecular weight ($\sim 2 \times 10^5$ Da).

The protein component of mucins consists of a single polypeptide chain in which amino acids are linked by peptide bonds.

MG1 comprises approximately 5,700 amino acids organized into three domains: the N-terminal domain, the central glycosylated domain, and the C-terminal domain, although the exact amino acid count varies between studies. The central glycosylated domain contains repeating units of 29 amino acids, with serine and threonine being the most prevalent. Amino acids within the C-terminal domain contribute to the formation of disulfide bonds that link mucin monomers into polymeric structures, which are "folded and packed" within the secretory granules of mucous glands. The stability of this packed mucin polymer is further maintained by divalent calcium ions. Upon secretion, calcium is replaced by monovalent sodium ions, increasing intramolecular osmotic pressure and leading to the hydration and extension of the high-molecular-weight mucin molecule, which then participates in the formation of the mucous gel.

MG2 is a salivary glycoprotein characterized by a low molecular weight ranging from 20 to 250 kDa. Its polypeptide chain consists of 357 amino acids, with the central region containing repeating sequences of 23 amino acids. Due to its low cysteine content, MG2 lacks the ability to form polymeric structures typical of high-molecular-weight mucins. Two variants of this mucin have been identified: MG2a and MG2b. While these variants do not differ in amino acid composition, they vary in the carbohydrate moiety, specifically in their sialic acid and fucose content. MG2a contains 14% sialic acid and 16% fucose, whereas MG2b contains 27% sialic acid and 7% fucose (Table 5.2).

Three groups of amino acids are notably abundant in the protein component of mucins.

1. Hydroxyamino acids, specifically serine and threonine.
2. Dicarboxylic amino acids, such as aspartic acid and glutamic acid.
3. Proline, glycine, and alanine.

Proline plays a crucial structural role due to its imino (NH) group, which disrupts the secondary helical conformation of the peptide chain. This disruption prevents the "folding" of mucin molecules into a globular shape, thereby facilitating the extensive binding of carbohydrate chains that are characteristic of mucins.

Glycine and alanine play a role in filling the bulk of the peptide chain due to their small size, but they do not significantly influence the nature or functional properties of mucins.

The free hydroxyl groups of the hydroxyamino acids, serine and threonine, serve as attachment sites for the carbohydrate side chains of the mature mucin molecule. Similarly, the dicarboxylic amino acids, aspartic acid and

glutamic acid, contribute by providing carboxyl (COOH) groups that facilitate the formation of bonds necessary for binding the numerous sugar moieties present in mucins.

In the side chains of mucins, N-acetylated hexosamines—specifically N-acetyl-glucosamine and N-acetyl-galactosamine—predominantly occupy the initial position (Figure 5.2). These hexosamines serve as crucial linkers, attaching the carbohydrate chains to the polypeptide backbone. Three principal types of bonds facilitate this connection: glucosidic, ester, and amide bonds. Glucosidic bonds, the most abundant, occur between hydroxyamino acids and hexosamines. Ester bonds form between dicarboxylic amino acids and the respective hexosamines, while amide bonds are established between glutamine or asparagine residues and hexosamines.

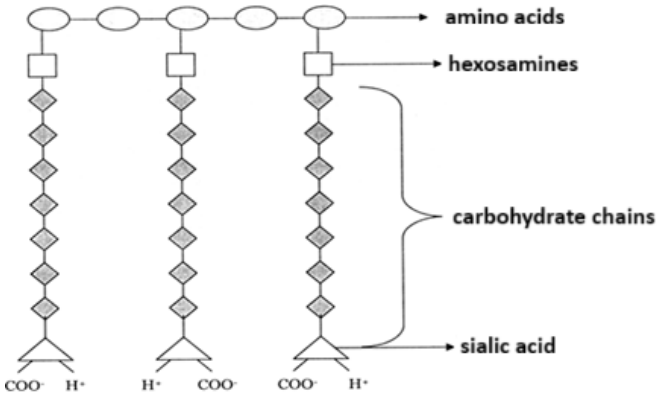


Figure 5.2: *Mucin structure and dissociation of the COOH group of sialic Acid*

The terminal position in the side chains of mucins is occupied by sialic acid.

The presence of sialic acid within the carbohydrate chains of sialomucins is critical for their functional properties and molecular stability. This significance stems from the inherent characteristics of sialic acid, particularly its low isoelectric point (pK = 2.6), which facilitates the dissociation of its carboxyl group even at pH values beyond the physiological range of saliva. This property leads to electrostatic repulsion among the similarly charged sialic acid residues, promoting maximal extension and spreading of the sialomucin molecules. Consequently, this ensures their optimal stability in solution and enables the full realization of their biological functions. Conversely, under conditions where

sialic acid dissociation is inhibited, such as exposure to stronger mineral acids at pH 2, the mucin molecules lose their electrification. This results in molecular "folding" and subsequent precipitation of the mucins.

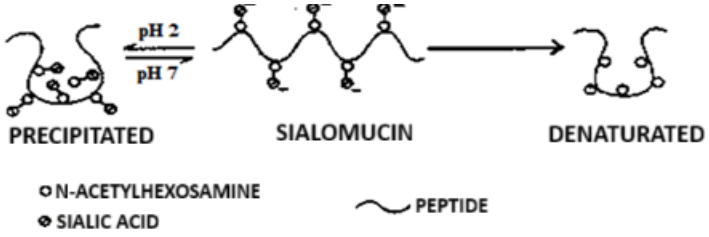


Figure 5.3: *Mechanism of salivary mucin deposition under the influence of low pH values and the enzyme sialidase.*

Table 5.1: Biochemical composition of MG1 and MG2 in saliva

Mucins	**MG_1**	**MG_2**
Molecular weight-Da	$>10^6$	2-2,5 x 10^5
Protein- mg/100mg	21,8	14,1
N-acetylgalactosamine - mg/100mg	11,8	13,7
N-acetylglucosamine- mg/100mg	17,6	23,4
Galactose - mg/100mg	24,9	26,8
Fucose - mg/100mg	11,3	13,6
Sialic acid - mg/100mg	3,9	3,8
Sulfates - mg/100mg	3,5	3,6
Fatty acids - mg/100mg	0,2	0,2

Table 5.2: Percentage of the constituent components of MG1 and MG2 in saliva

Mucins	**MG_1**	**MG_2**
Proteins	14,9 %	30,4 %
Carbohydrates Oligosaccharide chains (number) Sialic acid (number) Carbohydrates and their residue in the side chains (number)	 78,1 % 14 46 4-16	 68 % 170 67 2-7
Sulfates	7%	1,6 %
Fatty acids	unknown	unknown

MG1 is a salivary glycoprotein characterized by a high molecular weight exceeding 1,000 kDa. Its structural complexity surpasses that of MG2. MG1 exists as a tetramer, with individual monomeric mucin units interconnected through disulfide bonds (Figure 5.4).

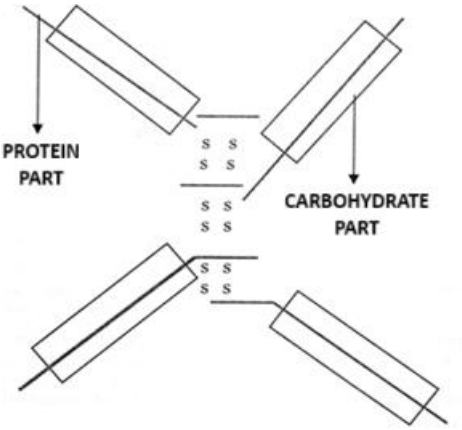


Figure 5.4: *Proposed model of the tetrameric mucin MG1*

Both types of salivary mucins interact with other salivary proteins, thereby contributing to the protection of oral structures. This interaction facilitates prolonged retention of salivary proteins within the oral cavity, which in turn enhances their functional efficacy.

It has been established that acidic and basic proline-rich proteins (PRP), statherins, and histatin 1 bind to the N-terminal domain of MUC7 (MG_2). These proteins exhibit antimicrobial properties. Through their interaction with mucin, their retention in the oral cavity is increased, thereby enhancing their antimicrobial function. Furthermore, mucins have been shown to facilitate the binding of secretory immunoglobulin A (sIgA) to the mucous pellicle, increasing its concentration in the oral environment and providing protective effects for the oral epithelium.

The interactions between mucins (MG1) and statherins, proline-rich proteins (PRP), and histatins can be disrupted by certain denaturing agents, suggesting that these interactions are mediated through hydrophobic, ionic, hydrogen, or van der Waals bonds. However, some complexes formed between MG1 and statherins or PRP resist disruption even under denaturing conditions, indicating that these complexes involve strong covalent bonds. Collectively, these findings suggest that salivary mucins function as transporters of antimicrobial peptides within the oral cavity gap, but at the same time protect the peptides from proteolytic degradation.

Salivary mucins have a number of important functions in maintaining oral homeostasis:

- provide a slimy appearance and viscosity of the salivary secretion;
- coat the oral mucosa with a protective film and thus protect it from maceration (damage) that would occur in a constantly moist environment;
- coat the bolus (bite) and enable its easier swallowing;
- facilitate tongue movements and speech;
- in the digestive tract (where mucin is present) they prevent the autodigestion of the intestinal mucosa, which would occur under the influence of numerous proteolytic enzymes from the digestive juices;
- they participate in the aggregation of bacteria, which enables their neutralization and inhibition of pathogenic activity;
- prevent the adherence of bacteria to the oral mucosa.

The coating role of mucin

Saliva maintains continuous contact with the tooth surfaces, and its components contribute to the formation of the acquired dental pellicle, a proteinaceous film on the enamel. Salivary mucins play a significant role in the

development of this thin protein layer on the tooth surface.

Mucins, present at concentrations of 30 to 40 mg/l, form a mucous gel that coats the oral mucosa. The extensive carbohydrate chains, whose sugar components are highly hydrated, bind water molecules intramolecularly within the gel. This structure effectively restricts the diffusion of macromolecules and significantly reduces the diffusion of low-molecular-weight molecules. The impregnation of the oral mucosa by mucins enhances its defensive capabilities by preventing the penetration of antigens from the external environment. This protective effect is mediated through interactions between the mucin and epithelial cells of the oral mucosa, which are established via numerous hydrophobic, hydrogen, and ionic bonds formed between the polyanionic mucin molecules and the epithelial cell surfaces. A receptor with a molecular mass of approximately 97 kDa has been identified in the membranes of epithelial cells of the oral mucosa, exhibiting a high affinity for salivary mucin. The interaction between this receptor and mucin is mediated primarily through the mucin's carbohydrate chains. Studies have demonstrated that deglycosylation of mucin reduces its binding to epithelial cells by up to 89%. The presence of this receptor in the epithelial cell membrane is critically important for maintaining the integrity and continuity of the mucous layer covering the oral mucosa. Disruption of the receptor-mucin interaction may result from the activity of bacterial enzymes, specifically glucosidases, which degrade the carbohydrate components of mucin. This enzymatic breakdown impedes the formation of the protective mucous layer, subsequently increasing the risk of lesions or wounds developing on the oral epithelial surface.

Antimicrobial effect of mucin

Recent studies have also demonstrated the antimicrobial properties of salivary mucins, implicating these glycoproteins in the nonspecific antimicrobial defense of the oral cavity (Figure 5.6). Under "*in vitro"* conditions, low-molecular-weight salivary mucins exhibit inhibitory effects against various fungi, including *Candida albicans* and *Cryptococcus neoformans*, as well as against gram-positive bacteria such as *Streptococcus mutans*, and gram-negative bacteria including *Porphyromonas gingivalis*, a known pathogen in periodontitis. Mucins play a crucial role in the prevention of dental caries by promoting the aggregation of bacteria within the oral flora. Numerous studies indicate that low-molecular-weight mucins are significantly more effective in bacterial aggregation compared to their high-molecular-weight counterparts. Notably, individuals susceptible to dental caries tend to exhibit higher concentrations of high-molecular-weight

mucins in their saliva, whereas increased levels of low-molecular-weight mucins have been observed in individuals resistant to caries. The two predominant salivary mucins, MG_1 and MG_2, are encoded by distinct genes. Evidence suggests that low-molecular-weight mucins may also be generated from high-molecular-weight mucins through the enzymatic activity of specific salivary proteases. These proteases cleave high-molecular-weight mucins, resulting in the formation of low-molecular-weight mucin fragments. It has also been found that the activity of salivary mucin proteases in caries-resistant individuals is significantly higher compared to the activity of this enzyme in caries-susceptible individuals.

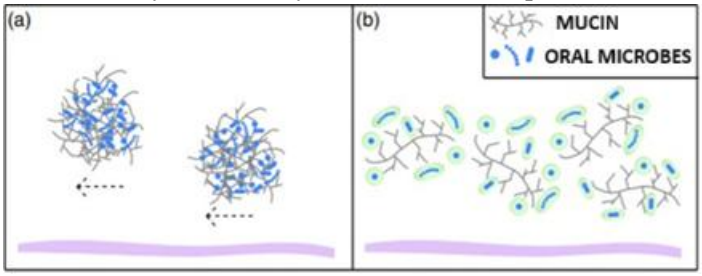


Figure 5.5: *Potential ways to protect against microbial colonization of the oral cavity. (a) Mucins in saliva agglutinate microorganisms, making them easier to remove upon swallowing. (b) Mucin reacts with bacterial adhesins, separating different bacterial colonies, reducing their virulence, and blocking their colonization in the oral cavity.*

By promoting the aggregation of oral bacteria, salivary mucins facilitate their clearance through swallowing and inhibit their adherence to the oral mucosa. (Figure 5.5). Certain mucin oligosaccharides share structural similarities with those present on the epithelial cells of the oral mucosa. Oral bacteria can adhere to these epithelial cells via specific adhesin molecules, enabling colonization. Because of the structural similarity between mucin oligosaccharides and those on epithelial cells, mucins interact with bacterial adhesins, effectively inhibiting bacterial colonization within the oral cavity (Figure 5.6). Additionally, aggregated bacteria are more readily eliminated from the oral environment through the cleansing action of saliva and the process of swallowing. The aggregation of bacteria by mucins, combined with their mechanical clearance through the rinsing action of saliva, constitutes a critical mechanism in preventing bacterial colonization and the initial infection of the oral mucosa. The pronounced ability of low-molecular-weight mucins to aggregate oral bacteria is attributed to their specific carbohydrate chains (particularly the binding of bacteria to the sialic acid residues present on these mucins). In addition to carbohydrate residues, the protein part of salivary mucin is also involved in interaction with bacteria. The N-terminal domain, which is rich in histidine and cysteine and plays a significant role in binding oral bacteria, is particularly important for this.

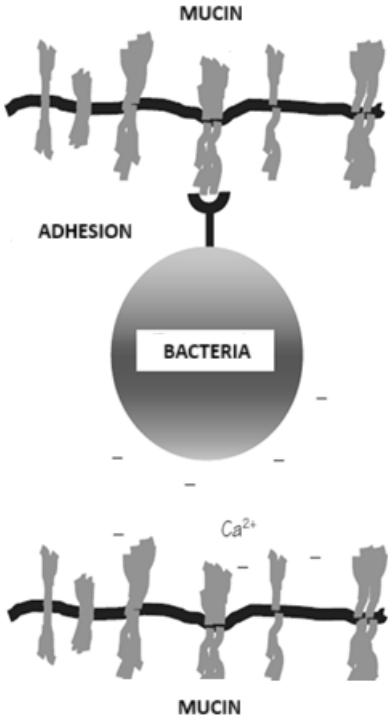


Figure 5.6: *The mechanism of bacterial aggregation by salivary mucin involves direct binding of mucin (sialic acid) to bacteria or indirect binding via Ca^{2+} ion.*

A peptide of approximately 20 kDa has been identified in the N-terminal domain of MG_2. This peptide is detectable in whole saliva but is absent in saliva from the submandibular and sublingual glands. This discrepancy is attributed to post-secretion modifications of mucins within the oral cavity, mediated by bacterial enzymes that degrade mucins into smaller peptide fragments exhibiting antimicrobial properties. Peptides rich in the amino acid cysteine demonstrate particularly significant antimicrobial activity.

The N-terminal domain of MG_2 plays a crucial role in its fungicidal activity. Peptides spanning amino acids 3–17 and 32–51 exhibit particularly strong antifungal effects. The peptide comprising amino acids 3–17 shares structural similarity with histatin 5. However, differences exist in the mechanisms underlying their fungicidal actions. Additionally, these two salivary biomolecules differ significantly in their degree of electrification, a factor that critically influences their interactions with microbial membranes. The peptide comprising amino acids 32–51 of MG_2 contains seven positively charged residues and no negatively charged residues, resulting in a net charge of +7. In comparison, histatin 5 possesses seven positively charged residues and two negatively charged residues, yielding a net charge of +5.

Histatin 5 binds to specific receptors on the membrane of the fungus *Candida albicans* and subsequently inhibits oxidative phosphorylation at the mitochondrial level. This inhibition leads to the production of free radicals, which cause oxidative damage to the fungal cell membrane. The MG_2 peptide comprising amino acids 32 to 51, being positively charged, electrostatically interacts with negatively charged components of the fungal membrane, such as phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, and ergosterol. It has been demonstrated that this MG_2 peptide penetrates the membrane and accumulates within the interior of the fungal cell.

Multiple studies have demonstrated that both major types of salivary mucins possess the capacity to inactivate HIV and inhibit its entry into human cells. An in vitro study confirmed that, even at low concentrations of MG_1 and MG_2, T-lymphocytes remained uninfected for up to three hours despite exposure to HIV. However, subsequent research has produced conflicting results. Further research is required to clarify the actual impact of salivary mucins on the HIV virus. Nevertheless, it is well established that HIV-infected individuals exhibit significantly lower concentrations of high-molecular-weight mucins compared to HIV-negative individuals.

SALIVARY AMYLASE (SELF-CLEANING OF THE ORAL CAVITY)

Salivary amylase, as a digestive enzyme, plays a vital role in the self-cleaning mechanisms of the oral cavity. This function is essential for maintaining oral health, given that the oral cavity represents the initial segment of the digestive tract, where food is moistened by saliva, mechanically processed through chewing, and subsequently swallowed.

If there is no self-cleaning of the oral cavity, numerous food particles will remain in the interdental spaces and in the fissures of the premolars and molars, and this has harmful consequences for the oral tissues (teeth and oral mucosa). These harmful consequences are due to:

- excessive proliferation of bacteria in the oral flora because nutrients (energy material) are available to them in greater quantities, and
- a multiple increase in the toxic products of bacteria that are released during their metabolic activity.

The listed harmful consequences, under physiological conditions, never occur (or do not occur to a large extent) because the human body responds to any stimulation of the oral receptors (chemical, physical or with other agents) by secreting stimulated mixed saliva. After all, this happens during eating, that is, chewing. The nutritional material present is dissolved in the secreted mixed saliva, and this is due to the percentage of the most significant component - water. Since water in the salivary secretion is represented by 99% (of the total content of saliva), in conditions of stimulated salivation, the ingested food will be dissolved more intensively. Considering the physiological process of salivary secretion (its swallowing and subsequent transport through the esophagus to the stomach) along with the solvent properties of mixed saliva, the self-cleaning function of the oral cavity becomes evident. This effect arises specifically from the dissolving and rinsing action of mixed saliva, which efficiently removes food particles from the oral cavity.

The oral cavity contains numerous retention areas (points where food is retained), such as interdental spaces and the fissures of premolars and molars. The self-cleaning effect in these regions is reduced, particularly in the upper jaw, as the dissolving and rinsing action of mixed saliva is less effective compared to that on the lower jaw teeth, which are more directly exposed to and continuously “bathed” in salivary secretions.

The flow rate of mixed saliva during stimulated secretion ranges from 0.2 to 5.7 ml/min. It is estimated that stimulated salivation can produce up to 30 times more saliva than unstimulated salivation. Consequently, the dissolving and rinsing effects of stimulated saliva are significantly more pronounced compared to those of unstimulated saliva.

In the period between meals, when mastication ceases and oral receptors are not significantly stimulated, unstimulated saliva is secreted at a rate approximately 30 times lower than that of stimulated saliva, ranging from 0.08 to 1.83 ml/min. Consequently, the dissolving and rinsing effects of unstimulated saliva are proportionally reduced. However, during this unstimulated phase, the concentration of the phosphate buffer increases on average by 2 to 3 times. As a result, the pH of mixed saliva decreases to 6.1. This acidification enhances the dissolving and rinsing properties of saliva, indicating that although the secretion rate is approximately 30 times lower, the self-cleaning effects of the oral environment are not diminished to the same extent—in fact, they are enhanced. Owing to its role in boosting the self-cleaning capacity of the oral cavity, the phosphate buffer is therefore regarded as a "cleaning buffer" of the oral environment.

The role of water (the most significant component of saliva) together with salivary buffer in the self-cleaning of the oral cavity is especially evident when processing water-soluble food substances. Among these, starch represents a significant dietary component in the modern human diet. Starch constitutes an integral component of bread, pasta, and similar food products. Due to its insolubility in water, starch particles tend to adhere and remain lodged within interdental spaces and the fissures of premolars and molars. The effective removal of these starch residues would be impossible without the presence of salivary amylase, an enzyme characterized by significant amylolytic activity.

Salivary amylase (ptyalin) is an enzyme responsible for hydrolyzing the α 1-4 glucosidic bonds within starch macromolecules. Starch granules consist of two polysaccharides: amylose and amylopectin. Amylose is composed of numerous glucose units linked primarily by α 1-4 glucosidic bonds. Salivary amylase catalyzes the hydrolytic cleavage of these bonds, resulting predominantly in the production of maltose molecules, which are highly soluble, along with smaller amounts of glucose.

Amylopectin exhibits a more complex structure compared to amylose, as its glucose units are linked not only by α 1-4 glucosidic bonds but also by α 1-6

glucosidic bonds. The hydrolysis of amylopectin by salivary amylase yields, in addition to a substantial amount of maltose and a small quantity of glucose, water-soluble oligosaccharides known as dextrins.

Starch particles retained in various oral retention sites are rapidly hydrolytically degraded by the enzymatic activity of salivary amylase, subsequently dissolved and removed through washing. The self-cleaning efficiency of the oral cavity is substantially enhanced by the presence of salivary amylase compared to when it relies solely on the dissolving and rinsing action of saliva's primary component—water. Through this self-cleaning function, salivary amylase significantly contributes to the prevention of oral diseases.

The preventive effect of salivary amylase is explained as follows:

- A starch grain is composed of a large number (n) of glucose molecules, each of which can be catabolized by oral bacteria into 2 molecules of lactate (lactic acid). Accumulation of retained starch particles in the oral cavity would thus lead to the production of a substantial amount of lactate from these glucose molecules. Given that lactic acid is a moderately strong organic acid (pH 2.9), when produced uncontrollably during the metabolic activity of dental plaque, causes a local decrease in pH below the critical value of 5.5. This acidification initiates the dissolution of hydroxyapatite crystals in tooth enamel, leading to tooth demineralization. However, such demineralization is prevented by the action of salivary amylase, which rapidly and effectively removes residual food particles. Consequently, oral bacteria are deprived of essential nutritional and energy sources.

Biochemical characteristics of salivary amylase

Salivary amylase is a monomeric protein structurally classified as an enzyme. Due to the presence of Ca^{2+} ions within its composition, it belongs to the group of metalloenzymes. The Ca^{2+} ion is crucial for maintaining the enzyme's molecular conformation, and its absence results in a reversible loss of enzymatic activity. Additionally, salivary amylase is among the few enzymes whose activity depends on the presence of chloride anions (Cl^-), which serve as activators.

Salivary amylase is present in two forms: glycosylated with a molecular mass of 62.000-63.000 Da and a non-glycosylated form with a molecular mass of 56.000-59.000 Da (Figure 5.7).

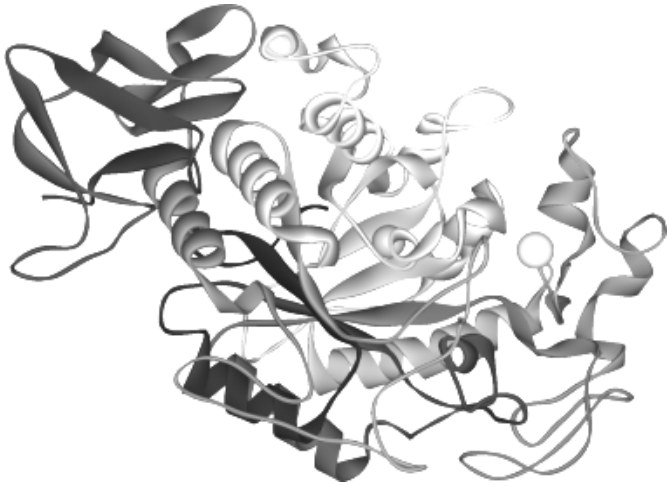


Figure 5.7: *Structure of salivary α-amylase*

The glycosylated form of salivary amylase contains carbohydrate side chains covalently attached to aspartic acid residues of the main polypeptide chain. This form also includes two sulfhydryl (SH) groups and four disulfide bonds. The polypeptide chain consists of 496 amino acids, among which the most prevalent are: (1) hydroxy-amino acids (threonine and serine), (2) dicarboxylic amino acids (aspartic acid and glutamic acid), and (3) aromatic amino acids (tryptophan and tyrosine). Similar to other globular proteins, salivary amylase exhibits an alpha-helix secondary structure, primarily stabilized by hydrogen bonds, which are particularly robust within the protein's hydrophobic core.

The site of synthesis of this enzyme is the glandular acini of the parotid and other salivary glands. It is quantitatively the most abundant protein in parotid saliva. Salivary amylase contributes 30% to the total protein composition of the parotid gland. In submandibular stimulated saliva, the concentration of this enzyme is 0.3 g/l, while in stimulated sublingual saliva, the concentration of salivary amylase is 0.25 g/l. In total stimulated saliva, salivary amylase is present at a concentration of 0.42 g/l.

There are multiple isoenzyme forms of this enzyme. In biochemistry, the term isoenzyme refers to distinct electrophoretic variants of the same enzyme that catalyze the same type of enzymatic reaction involving an identical substrate. Similar to other salivary proteins, salivary amylase is subject to varying degrees of proteolytic activity within the oral cavity. Recent studies have demonstrated the presence of more than 500 isoforms (isoenzymes) of salivary amylase in saliva. The presence of such a large number of isoforms is attributed to post-

translational modifications of the enzyme, resulting from physiological proteolytic processes occurring in saliva.

Each isoenzyme of salivary amylase is characterized by a distinct isoelectric point and exhibits varying enzymatic activity depending on the pH of the oral environment. Throughout the day, physiological fluctuations in oral pH range from 6,1 to 7,8. The activity range of salivary amylase extends from pH 5,8 to 6,9. Consequently, at any pH value within this range, certain isoenzyme forms remain active and contribute to the enzymatic degradation of residual starch particles, thereby supporting the self-cleaning function of the oral cavity.

Salivary amylase also plays a significant role in the metabolism and colonization of oral bacteria involved in the formation of dental biofilm. Its presence, along with other salivary proteins, has been identified in the dental pellicle. Within this structure, salivary amylase functions as a receptor facilitating the adhesion of oral bacteria during biofilm development. This interaction may be beneficial for maintaining oral homeostasis, as the attached bacteria become more susceptible to local defense mechanisms. However, under conditions of inadequate oral hygiene, the bacterial-binding capacity of amylase becomes disadvantageous, as it promotes the accumulation of cariogenic and periodontopathogenic microorganisms.

This protein, in its enzymatic capacity, is capable of binding to bacterial endotoxins, as well as to a wide range of other toxins that significantly contribute to inflammatory tissue degradation.

PROLINE-RICH PROTEINS (PRPs)

Proline-rich proteins (PRPs) are salivary proteins characterized by a high content of the amino acid proline. Based on the presence of a carbohydrate moiety and their electrical charge, PRPs are classified into three groups:

- acidic PRPs with a molecular mass of ≤ 16 kDa;
- basic PRPs with a molecular mass ranging from 6 to 9 kDa;
- glycosylated PRPs with a molecular mass of 39 kDa.

➢ Acidic PRPs belong to the class of salivary proteins exclusively present in salivary secretions. The most significant acidic PRPs detected in parotid and submandibular saliva are PRP-1 (protein C) and PRP-3 (protein A).

- PRP-1 (protein C) consists of a single polypeptide chain comprising 150 amino acids, with proline, glutamine, and glycine being the most abundant amino acids in this chain.
- PRP-3 (protein A) consists of 106 amino acids. It is formed by proteolytic cleavage of PRP-1 between the amino acid arginine (at position number 106 in the polypeptide chain) and the amino acid glycine (at position number 107 in the polypeptide chain).

➢ Apart from saliva, basic PRPs are also present in nasal and bronchial secretions. The parotid glands produce basic PRPs, while the submandibular and sublingual salivary glands do not participate in the production of this class of proteins. They are built with proline-rich sequences of varying lengths, and the amino acids arginine and lysine are also present in the peptide chains. Basic PRPs adhere to the tooth surface, play a role in binding various oral bacteria, as well as binding various polyphenols.

➢ Glycosylated proline-rich proteins (PRPs) are glycoproteins composed of protein and carbohydrate components. The protein portion constitutes 60% of their total mass and consists of a polypeptide chain predominantly containing the amino acids proline, glycine, and glutamic acid. The presence of basic amino acids (lysine and arginine) in the protein segment, combined with the absence of sialic acid in the carbohydrate portion, imparts a basic character to these glycoproteins, reflected by an isoelectric point greater than 8.2. Due to their basic nature, these glycoproteins carry a positive charge and migrate toward the cathode in an electric field. The carbohydrate component, comprising 40% of the molecule, consists of fucose (4.5%), N-acetylgalactosamine (6.9%), galactose (7.5%), N-acetylglucosamine (21.5%), and mannose (5%). Notably, sialic acid is absent from the carbohydrate portion of glycosylated proline-rich proteins (Figure 5.8).

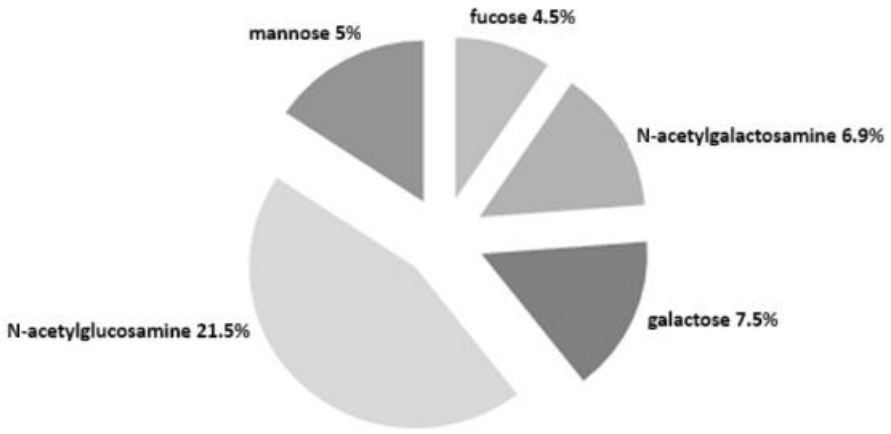


Figure 5.8: *Components of the carbohydrate part of glycosylated PRPs (40%)*

The role of proline-rich proteins (PRPs) in maintaining oral homeostasis is significant. Their functions include:

1. protection of tooth enamel,
2. binding of dietary tannins,
3. antimicrobial activity,
4. antiviral activity, and
5. formation of the acquired dental pellicle.

Tooth enamel protection

The stability of hydroxyapatite crystals on tooth enamel depends on the degree of saturation of saliva with calcium salts and phosphate salts. The saturation of saliva with the same minerals, as well as the minerals that participate in the construction of tooth enamel, reduces the possibility of dissolving tooth enamel in saliva (which is a universal solvent since 99% of this secretion is represented by H_2O). However, oversaturation with calcium and phosphate ions poses the risk of undesirable precipitation of calcium-phosphate salts. To prevent spontaneous deposition of these salts, salivary proteins such as statherins and acidic proline-rich proteins (PRPs) inhibit non-physiological precipitation on the

tooth enamel surface.

In the absence of these inhibitors, deposits would accumulate on the tooth enamel and within the excretory ducts of the salivary glands. Such deposits would subsequently lead to instability and increased solubility of hydroxyapatite crystals. The inhibitory effect of acidic PRPs (on the precipitation of calcium and phosphate salts) is enabled by their strong affinity for calcium and hydroxyapatite.

Acidic PRPs bind calcium via phosphate groups located at the N-terminal region of the polypeptide chain, thereby serving as “reservoirs” of this electrolyte in saliva. Salivary proteins, particularly PRPs, play a significant role in the remineralization processes of tooth enamel. Under suitable conditions in the oral cavity, these calcium “reservoirs” may be utilized to repair the damaged surface layers of this dental tissue. It has also been demonstrated that the phosphate groups of the amino acid serine present in PRPs contribute to the binding of acidic PRPs to the negatively charged hydroxyapatite. This binding occurs indirectly through the mediation of Ca^{2+} ions. As a divalent ion, Ca^{2+} forms one bond with the negatively charged enamel surface and another with the acidic PRPs. By binding to the tooth enamel surface, acidic PRPs contribute to the formation of the acquired dental pellicle, which plays an important role in the mechanical protection of this dental tissue.

Binding of dietary tannins

Tannins are polyphenolic compounds found in plant-based foods. Salivary proteins such as proline-rich proteins (PRPs) and histatins are capable of forming complexes with tannins, thereby exerting a preventive and protective effect against their potentially toxic and carcinogenic properties. Tannins can have several adverse effects on human tissues, including interference with electrolyte absorption in the gastrointestinal tract, inhibition of digestive enzyme activity, and pathological effects on the lungs and liver. The capacity of basic PRPs to bind dietary tannins is attributed to the high abundance of proline-rich amino acid sequences within their peptide structure. Furthermore, the high glycine content in PRP molecules facilitates the formation of large, open binding surfaces for tannins. The formation of insoluble PRP–tannin complexes involves hydrogen bonding and hydrophobic interactions. Basic PRPs play a key role in tannin binding, whereas glycosylated PRPs participate in this process only after

deglycosylation (removal of the carbohydrate moiety). The formation of PRP–tannin complexes stimulates salivary secretion, which in turn elevates the concentration of basic PRP (originating from parotid saliva) thereby enhancing the formation of insoluble complexes with tannins and mitigating their potential toxic effects.

The formation of complexes between basic PRPs and tannins gives rise to an astringent sensation in the oral cavity. This sensation, commonly experienced after the consumption of red wine, is a tactile perception caused by a reduction in lubrication within the mouth.

Antimicrobial effect

A large number of salivary proteins and glycoproteins contribute significantly to the aggregation of oral bacteria and facilitate their removal from the oral cavity. Proline-rich proteins (PRPs) also perform this function. However, PRPs in solution (e.g., in saliva) exhibit low bacterial-binding capacity, as their bacterial-binding receptors are masked. The ability of PRPs to bind bacteria increases markedly once they are adsorbed onto the hydroxyapatite surface of tooth enamel. Upon incorporation into the acquired dental pellicle, PRPs undergo conformational changes that expose previously hidden binding sites (so-called cryptotopes) These cryptotopes may include low-molecular-weight molecules such as short PRP-derived peptides (e.g., proline-glutamine-glycine-proline-glutamine), which serve as bacterial adhesion sites. The fimbriae of *Porphyromonas gingivalis* have been shown to bind to these peptides. Additionally, it has been confirmed that these peptides can also bind to *Fusobacterium nucleatum*, another virulent oral bacterium. Bacterial binding to PRPs can also occur indirectly, mediated by divalent calcium ions (Ca^{2+}), which intercalate between the phosphate groups of PRPs and the bacterial surface.

Furthermore, in vitro studies have demonstrated that basic PRPs can reduce the virulence of *Herpes simplex virus* type 1. There is also evidence suggesting that basic PRPs may inhibit the invasion of HIV.

Formation of the acquired dental pellicle

Basic PRPs are positively charged and can bind directly to the negatively charged tooth enamel surface. The binding of basic glycoproteins to the tooth enamel surface occurs most intensively during mastication (chewing) when stimulated saliva is secreted. It has been proven that the amount of PRP in saliva

increases with stimulation of salivary secretion. When these glycoproteins bind to the surface of the tooth enamel, an acquired organic layer called the dental pellicle is formed on the biting surfaces of the teeth. It has a lubricating effect; namely, the formed dental pellicle reduces the effect of excessive friction on the teeth that occurs during chewing food. In this way, the tooth enamel is partially

protected from excessive loss (attrition) during mastication. However, apart from the aforementioned positive property of the dental pellicle, it serves as an organic basis for the attachment of bacteria from the oral flora and the creation of bacterial biofilm.

LINGUAL LIPASE

Lingual lipase is produced by small serous glands on the tongue called Von Ebner's glands. This enzyme begins the breakdown of triglycerides in the mouth by breaking the ester bonds between fatty acids and glycerol. However, the efficiency of this enzyme is low due to the short residence time of food in the mouth and the absence of emulsifiers, such as bile salts.

SALIVARY CARBONIC ANHYDRASE (CA)

Salivary carbonic anhydrase is a protein by structure and an enzyme by function, essential for maintaining the physiological pH of saliva. An isoenzyme, carbonic anhydrase VI (CA VI), is present in saliva, with a molecular mass of 42 kDa. Besides amino acids, this protein contains two oligosaccharide chains linked to the main polypeptide chain via O-glucosidic bonds.

Studies indicate that CA VI is localized exclusively in the secretory granules of acinar cells of the parotid and submandibular glands, from where it is secreted into saliva. Salivary carbonic anhydrase constitutes 3% of the total protein content in parotid saliva.

The secretion of CA VI fluctuates throughout the day. Its concentration is lowest during the night and peaks immediately after awakening. A positive correlation has been observed between the activity of salivary amylase and the concentration of CA VI in saliva, suggesting that both enzymes are secreted via similar mechanisms and are localized within the same secretory granules of the acinar cells in the salivary glands.

The primary function of salivary carbonic anhydrase is to maintain

physiological pH levels in the oral cavity. Specifically, it contributes to the production of bicarbonate ions, which are essential for the buffering capacity of saliva. These bicarbonate ions (HCO_3^-) are of endogenous origin. Carbon dioxide, a metabolic byproduct of organic molecule degradation in the body, reacts with water to form carbonic acid under the catalytic action of salivary carbonic anhydrase. This carbonic acid subsequently dissociates into a bicarbonate anion and a hydrogen cation.

$$CO_2 + H_2O \longrightarrow H_2CO_3 \longrightarrow HCO_3 + H^+$$

Due to its aforementioned functions, this enzyme plays a crucial role in preventing the demineralization of tooth enamel. It is the first protein that has been scientifically proven to be associated with dental caries. A negative correlation has been observed between the DMFT index (decayed, missing, and filled teeth) and the concentration of CA VI in saliva.

Salivary carbonic anhydrase is also a component of the dental pellicle. Within this pellicle, CA VI catalyzes the reaction between salivary bicarbonate ions and hydrogen ions (byproducts of bacterial metabolism) converting them into carbon dioxide and water. Through this mechanism, salivary CA VI protects the enamel from acid exposure resulting from bacterial metabolic activity (Figure 5.9).

$$HCO_3 + H^+ \xrightarrow{\text{CA VI}} CO_2 + H_2O$$

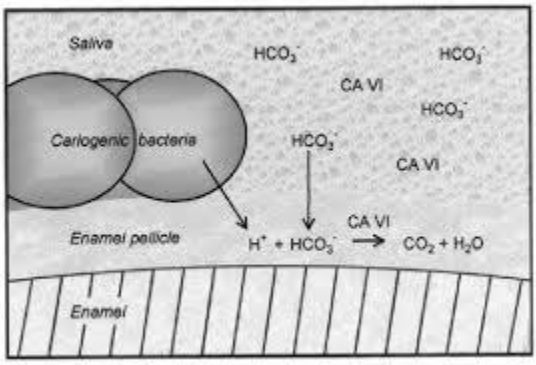


Figure 5.9: *The action of CA VI in the dental pellicle*

The epithelial cells of the oral mucosa and the esophagus are continuously exposed to various chemical and physical stimuli during food intake. One of the key protective functions of saliva is to safeguard the mucosa

of the upper segments of the digestive tract. Numerous salivary proteins (including mucins, amylase, cystatins, proline-rich proteins (PRP), and salivary carbonic anhydrase) associate with the surface of the epithelial cells lining the oral mucosa. This association forms a protective layer known as the "mucosal pellicle," which serves as a barrier shielding the oral mucosa from mechanical abrasion, chemical irritation, and the deleterious effects of proteolytic enzymes released by bacteria and neutrophilic leukocytes. Salivary bicarbonates play a crucial role in maintaining physiological pH values at the surface of the epithelial cells lining the oral and esophageal mucosa, thereby protecting these tissues from the effects of acidic by-products of bacterial metabolism and from hydrochloric acid originating from gastric reflux. Given that the formation of salivary bicarbonates depends on the catalytic activity of salivary carbonic anhydrase, it can be concluded that this enzyme is also essential for the protection of the mucosal surfaces in the upper regions of the digestive tract.

ORAL PEROXIDASE

Oral peroxidase is the most important enzymatic antioxidant in saliva. This enzyme is composed of two peroxidase enzymes: salivary peroxidase (80%) and myeloperoxidase (20%).

Salivary peroxidase is secreted by the major salivary glands, with the parotid gland being the primary source. This enzyme contains selenium in its active center. The role of salivary peroxidase lies in its ability to reduce hydrogen peroxide (H_2O_2), a product of oral bacterial metabolism, in the presence of thiocyanate ions (SCN^-). Thiocyanates are a component of saliva and act as electron donors. Their function is somewhat similar to that of reduced glutathione, which serves as an electron donor in other biological systems. Thiocyanates originate from hydrogen cyanide (HCN) found in tobacco smoke. HCN is detoxified in the liver and then transported by the bloodstream to the salivary glands. There, it enters the salivary secretion through ultrafiltration (Figure 5.10).

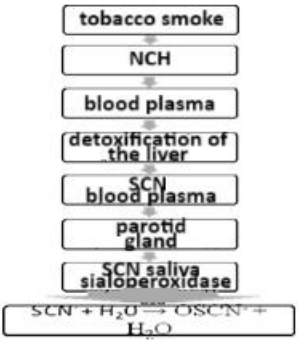


Figure 5.10: *The action of salivary peroxidase (sialoperoxidase)*

Salivary peroxidase catalyzes the reaction between H_2O_2 and thiocyanate ions. The products of this reaction are hypothiocyanous acid (HOSCN) and hypothiocyanate ions ($OSCN^-$). These compounds exhibit antibacterial activity. Their antibacterial effect is mediated through interaction with the sulfhydryl groups (SH) of bacterial enzymes involved in glycolysis, including hexokinase, aldolase, and pyruvate kinase. Beyond its role in the nonspecific antibacterial defense of the oral cavity, salivary peroxidase also facilitates the effective removal of H_2O_2 from the oral environment.

CYSTATINS

Cystatins are proteins characterized chemically by their function as inhibitors of cysteine protease enzymes. The cystatin family comprises proteins that differ in primary structure, molecular size, and localization. Based on the length of the polypeptide chain and the presence or absence of disulfide (S-S) bonds, cystatins are classified into three families: I, II, and III.

Family I (stefins) includes cystatins A and B. These are the smallest proteins within the cystatin group, with a molecular mass of approximately 11 kDa. They consist of a single polypeptide chain composed of about 100 amino acids. They lack disulfide bridges and carbohydrate moieties. Their localization is primarily intracellular, within the cytoplasm, although they can be detected in low concentrations in various extracellular fluids. Cystatin A is predominantly localized in epithelial cells and polymorphonuclear leukocytes, whereas cystatin B is found in nearly all tissues.

Family II includes cystatins S, C, D, G, SN, and SA (Figure 5.11). This group also comprises the recently identified cystatins E/M and F (referred to as

leukocystatins), which share a similar structure with other cystatins in this family. Like the previous family, these cystatins possess a single polypeptide chain of approximately 120 amino acids and contain disulfide bridges in the C-terminal region. Notably, cystatin F has a disulfide bridge located in the N-terminal region. Most cystatins in this family are unglycosylated, except for cystatin F, which contains two glycosylation sites, and cystatin E/M, which has one glycosylation site in the N-terminal part (108). Human cystatins S and SA are partially phosphorylated, in contrast to cystatin SN. Various forms of cystatin S isolated from saliva exhibit phosphorylation at sites within the N-terminal region (positions 2, 98, 111, 114). Family II cystatins are extracellular proteins, present in high concentrations in biological fluids and secretions, including saliva, tears, urine, seminal plasma, and cerebrospinal fluid.

Family III (kininogens). Members of this family possess a molecular mass ranging from 50 to 80 kDa. Structurally, they are glycoproteins. Three types of kininogens have been identified: kininogen L (low molecular mass), kininogen H (high molecular mass), and kininogen T (also known as thiostatin). Kininogens are multifunctional proteins recognized as precursors of vasoactive peptides called kinins. The kinin sequence is located in the C-terminal region of the kininogen molecule and is released upon proteolytic degradation of this cystatin.

Figure 5.11: *Cystatine C structure*

The primary biological function of cystatins is the inhibition of cysteine protease enzymes, which are present intracellularly (endogenous cysteine proteases). Proteases constitute a crucial group of enzymes essential for cellular and organismal function. These enzymes catalyze the cleavage of peptide bonds within proteins or peptides. Based on the chemical group responsible for catalysis, proteases are categorized into four main classes: serine, cysteine, threonine, and metalloproteases. Proteases play a pivotal role in tissue protein metabolism, physiological protein degradation, and tissue repair processes. Protease activity is tightly regulated at multiple levels to prevent unwanted proteolysis and cellular or tissue damage. One of the principal mechanisms for controlling protease activity is through protein inhibitors. As inhibitors of cysteine proteases, cystatins protect tissue proteins from unphysiological degradation. Additionally, cystatins safeguard tissues against the proteolytic effects of cysteine proteases derived from viruses, bacteria, and parasites (exogenous cysteine proteases).

Cystatins inhibit cysteine proteases reversibly, following the principle of competitive inhibition. Specifically, cystatins indirectly block the active site of these enzymes, thereby preventing the enzyme from binding to its substrate (protein) and subsequent degradation. Cystatins bind to the active site of cysteine proteases, forming a stable bimolecular complex that maintains the enzymes in an inactive state for hours to weeks.

Cystatins SN, SA, S, and C are present in human saliva, with recent studies also demonstrating the presence of cystatin D. The predominant cystatin in saliva is cystatin S. Experimental evidence indicates that cystatin C, although less abundant in saliva compared to cystatin S, plays the most significant role in the inhibition of cysteine proteases by saliva (Table 5.3).

Table 5.3: Cystatin concentration (μmol/l) in different types of salivary secretion

Cystatin	Mixed saliva	Submandibular saliva	Parotid saliva
C	0,09-0,1	0,11	0,03
D	0,27	-	-
S	7,3-8,2	12,5	0,007
SA	4-5	-	-
SN	2,8	6,3	0,08
E/M	-	-	-
F	-	-	-

In addition to their primary function of inhibiting cysteine proteases, these salivary proteins perform other important roles, such as:

- Phosphorylated forms of cystatin S are components of the acquired dental pellicle.

- Antimicrobial properties of cystatins. Numerous studies have demonstrated that cystatins can inhibit the growth and proliferation of bacteria. The inhibition of Porphyromonas gingivalis growth is not related to the enzyme-inhibitory function of cystatins, but rather to specific peptides within the cystatin family. Furthermore, cystatins exhibit antiviral activity, as evidenced by their in vitro inhibition of the replication of certain viruses, including coronavirus, poliovirus, and adenovirus.
- Anti-inflammatory effect in in vitro conditions proved that cystatins can regulate the production of inflammatory mediators-cytokines by gingival fibroblasts.

STATHERIN

Statherin is a small protein based on its molecular mass. It belongs to the group of acidic proteins, with a peptide chain composed of 43 amino acids. It is also referred to as a proline-rich or tyrosine-rich phosphoprotein due to its high content of the amino acids proline and tyrosine. One third of the amino acid residues in the polypeptide chain are negatively charged, including aspartic acid, glutamic acid, and phosphoserine. The remaining two thirds of the amino acid residues are hydrophobic and polar amino acids such as glutamine, proline, and tyrosine (with the exception of glutamic acid). This salivary protein is found in both parotid and submandibular saliva. The name "statherin" derives from the Greek word "statheropio," meaning "to stabilize." The concentration of statherin varies individually across different types of salivary secretion (Table 5.4).

Table 5.4: Concentration of statherin in different types of salivary secretion

	Stimulated mixed saliva	**Stimulated parotid saliva**	**Stimulated submandibular and sublingual saliva**
Statherin concentration (μg/ml)	4,30 ± 3,11	95,99 ± 64,90	73,60 ± 45,80

Stathin, as a multifunctional protein present in saliva, holds significant importance for oral homeostasis. The primary role of this salivary component is to prevent the deposition of calcium phosphate salts in the drainage ducts of the salivary glands. This function reduces the risk of calculus (stone) formation within these structures. Specifically, saliva is saturated with minerals (particularly calcium and phosphates, which are the main inorganic constituents of hard dental tissues) playing a crucial role in preserving the integrity of tooth enamel. However, this mineral saturation of saliva also poses the risk of unwanted precipitation and stone formation. This phenomenon is particularly prone to occur in the drainage ducts of the salivary glands. Statherin is responsible for mitigating this risk by binding Ca^{2+} ions, thereby preventing their precipitation.

Statherin also exhibits a strong affinity for calcium phosphate minerals, such as hydroxyapatite, indicating its role in regulating the solubility of tooth enamel. This salivary protein inhibits spontaneous precipitation and crystal growth in calcium phosphate-supersaturated fluids like saliva. The N-terminal region of the statherin polypeptide chain (amino acid residues 1–15) is primarily responsible for this inhibitory effect, likely due to its high affinity for calcium ion binding.

6 SALIVA PROTEINS INVOLVED IN SPECIFIC IMMUNOLOGICAL DEFENSE

IMMUNOGLOBULIN FEATURES

Basic structure of antibodies

SALIVARY IMMUNOGLOBULIN A (sIgA)

CHAPEROKINES (HSP70/HSPA)

IMMUNOGLOBULIN FEATURES

Immunoglobulins (Ig), commonly known as antibodies, are biologically active molecules produced by B-lymphocytes or plasma cells. Biochemically, they are classified as glycoproteins and migrate within the γ-globulin fraction when subjected to an electric field. Their composition consists of approximately 82–96% protein and 4–18% carbohydrate. The protein portion is responsible for all biological functions of immunoglobulins.

Antibodies interact with antigens (epitopes) through specific antigen-binding sites (paratopes). Antigens stimulate the production of antibodies, which subsequently bind to their respective antigens with high specificity.

All antibodies share common features: structural characteristics, antigen binding, and participation in a limited number of effector functions. The antibodies produced in response to a specific antigen are diverse. Most antigens are complex and contain many different antigenic determinants, and the immune system typically responds by producing antibodies to several epitopes on the antigen. This response requires the recruitment of multiple B-cell clones. Their products are monoclonal antibodies that specifically bind to individual antigenic determinants. The polyclonal and heterogeneous serum antibody response is composed of all these monoclonal antibodies.

Basic structure of antibodies

Blood can be separated by centrifugation into two main components: a liquid fraction and a cellular fraction. The liquid portion is known as plasma, while the cellular fraction consists of erythrocytes, leukocytes and platelets. Plasma contains all the soluble small molecules and macromolecules present in blood, including fibrinogen and other proteins essential for blood coagulation. Once blood coagulates, the remaining liquid portion is referred to as serum. Importantly, antibodies are found in the serum. This was first demonstrated by Tiselius and Kabat in 1939, who conducted an experiment in which they immunized rabbits with ovalbumin (a protein found in egg white). They then analyzed the serum from the immunized rabbits using electrophoresis, dividing it into two aliquots (i.e., samples loaded into wells in a gel).

Electrophoresis of a sample of blood serum revealed four distinct peaks, corresponding to albumin, alpha globulins, beta globulins, and gamma globulins (Figure 6.1). In the experiment, the portion of the serum that reacted with ovalbumin formed a precipitate, which was subsequently removed. The

remaining serum proteins, those that did not react with the antigen, were then subjected to electrophoresis. When the electrophoretic profiles of the original serum and the reacted serum were compared, a marked decrease in the gamma globulin fraction was observed in the latter. This reduction indicated that the antibodies responsible for reacting with the antigen were located in the gamma globulin region. As a result, these proteins were designated as immunoglobulins to differentiate them from other proteins that may also be present within the gamma globulin fraction. According to the early experiments by Tiselius and Kabat, serum proteins were divided into three major non-albumin peaks: alpha, beta, and gamma. Today, it is also known that although immunoglobulin G (the main class of antibodies) is found predominantly in the gamma-globulin fraction, significant amounts of it as well as other important antibody classes have also been detected in the alpha and beta fractions of serum.

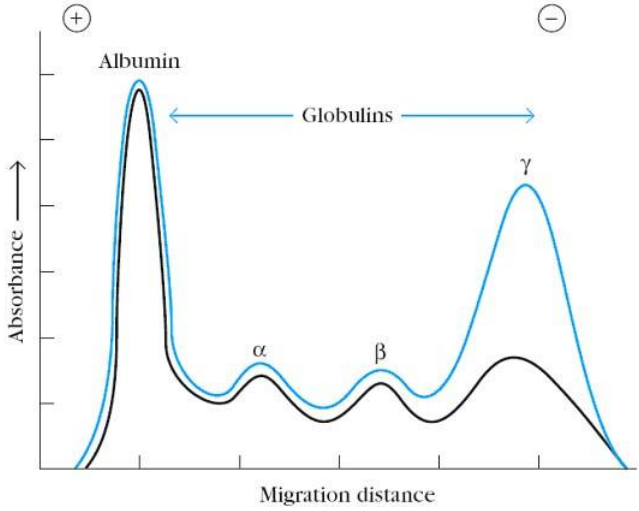


Figure 6.1: *Experimental illustration showing that antibodies are found in the gamma-globulin fraction of serum proteins*

The fundamental structural unit of immunoglobulins consists of two pairs of polypeptide chains (two heavy chains and two light chains) (Figure 6.2). The general formula of immunoglobulins is denoted as H_2L_2, where **H** represents the heavy chains and **L** represents the light chains. Numerous disulfide bonds (S–S bonds) are located within the individual chains. In addition, disulfide bonds are also present between the chains. The presence of intrachain disulfide bonds facilitates the formation of discrete domains within the immunoglobulin chains. A domain is defined as a segment of the immunoglobulin polypeptide chain capable of folding independently of the remaining structure, contributing to the compact and stable conformation of the molecule.

Light chains of immunoglobulins consist of two domains located in the N-terminal (NH_2) region. The first 110 amino acids of the light chain form the variable domain. This variable domain of the light chain pairs with the variable domain of the heavy chain to form the antigen-binding site, also known as the paratope. The variable domain is succeeded by the constant domain of the light chain. Based on the amino acid sequence of the light chain, two types are distinguished: kappa and lambda light chains.

Heavy chains of immunoglobulins also begin with a variable domain composed of approximately 110 amino acids. This domain, together with the variable domain of the light chain, forms the paratope. Following the variable domain, constant domains are arranged in sequence along the heavy chain. IgG, IgA, and IgD each contain three constant domains in their heavy chains, whereas IgM and IgE contain four. The specific sequence and structure of the amino acids in the constant region of the heavy chain determine the class or subclass of the immunoglobulin molecule (IgG_1, IgG_2, IgG_3, and IgG_4, IgA_1 and IgA_2). Upon enzymatic cleavage by papain, the immunoglobulin molecule is “cleaved” just above the intrachain disulfide bond (S-S) in the hinge region (Figure 6.3). This process yields three fragments: two Fab (fragment antigen-binding) fragments and one Fc (fragment crystallizable) fragment. The primary biological function of the Fab fragments is antigen binding. The Fc fragment facilitates the transplacental transport of immunoglobulins (IgG). In addition, this portion of the immunoglobulin is involved in cytotoxic reactions and in the activation of complement via the classical pathway.

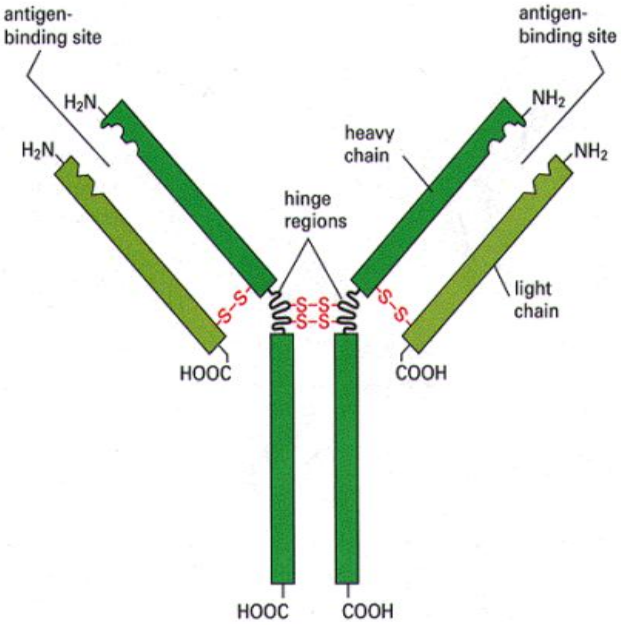


Figure 6.2: *Schematic representation of a typical immunoglobulin molecule.*

Immunoglobulins are made up of four polypeptide chains, two identical heavy chains and two identical light chains. The two antigen-binding sites are identical and are formed between the N-terminal ends of the light and heavy chains. The tail or constant (Fc) is made up of the heavy chains only.

The hinge region of the immunoglobulin molecule constitutes a structural domain targeted by proteolytic enzymes, such as papain and pepsin. This region is characterized by a high content of the amino acids proline and cysteine. Cysteine residues facilitate the formation of disulfide bonds, which are critical for molecular stability. Proline residues contribute to the spatial separation of the immunoglobulin fragments (Fab and Fc) and confer molecular flexibility to the individual domains of the immunoglobulin molecule.

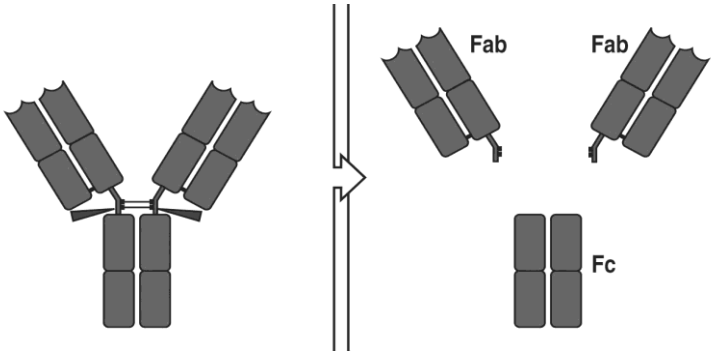


Figure 6.3: *Proteolytic cleavage of an immunoglobulin molecule with the enzyme papain*

Based on the arrangement of amino acids in the constant domains of the heavy chains, five classes of immunoglobulins are distinguished (Figure 6.4):

1. immunoglobulin G- IgG
2. immunoglobulin A- IgA
3. immunoglobulin M- IgM
4. immunoglobulin D- IgD
5. immunoglobulin E- IgE

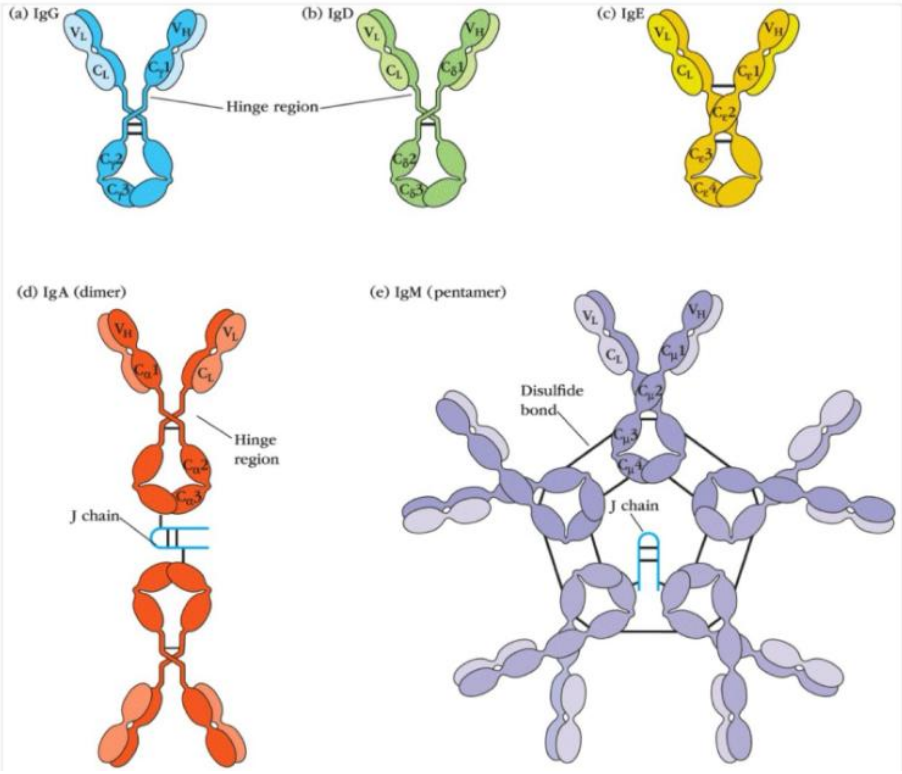


Figure 6.4: *General structure of the five major classes of antibodies. It is seen that IgG, IgA, and IgD have heavy chains with four domains and a hinge region, while IgM and IgE have five domains but no hinge region. The polymeric forms of IgM and IgA contain a J chain, which is linked by two disulfide bonds to the Fc region of two monomers. Serum IgM is always a pentamer; most IgA in serum is present as a monomer, but it is also found as a dimer, trimer, and rarely as a tetramer.*

Based on where they are found, immunoglobulins are divided into:

1. membrane (IgM, IgD)
2. secretory (present in various types of secretions)

The immunoglobulin classes found in secretions such as saliva include IgA (the predominant class), IgM, and IgG. Together, these form the secretory immunoglobulin system. A portion of these immunoglobulins, particularly IgA and to some extent IgM, are synthesized locally. Immunoglobulin G in secretions (saliva) reaches the oral cavity by filtration through the sulcus epithelium.

SALIVARY IMMUNOGLOBULIN A (sIgA)

Although IgA constitutes approximately 10–15% of the total immunoglobulins in serum, it is the predominant class of immunoglobulins in external secretions such as breast milk, saliva, tears, and mucus from the bronchial, urogenital, and gastrointestinal tracts. Immunoglobulin A (IgA) serves as the principal mediator of local specific immunity in the oral mucosa of humans. In the serum, IgA exists in a monomeric form, composed of two light and two heavy chains. The serum IgG to IgA ratio is 4:1 in favor of IgG, whereas in mucosal secretions this ratio shifts to 1:1. Within these secretions, IgA is primarily found in a dimeric form, consisting of two monomeric units linked together. The two monomers of IgA are joined by a specific peptide known as the J-peptide (Figure 6.5). The J-peptide functions to link immunoglobulin monomers into polymeric forms through stable covalent disulfide (S-S) bonds. This peptide, like the immunoglobulin itself, is synthesized by plasma cells. A distinctive feature of secretory IgA is the presence of an additional molecular component known as the secretory component (SC).

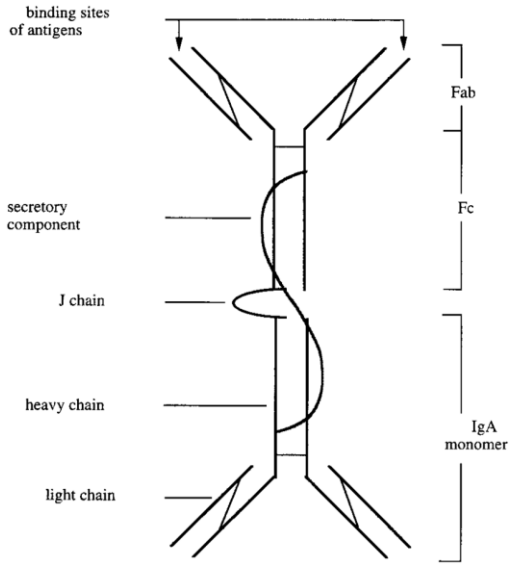


Figure 6.5: *Schematic representation of sIgA. sIgA consists of two IgA monomers linked by a J chain and a secretory component. The J chain and secretory component are linked by disulfide bonds to the Fc fragment of the IgA molecule. Each IgA monomer consists of two light chains and two heavy chains linked by covalent disulfide bonds.*

The secretory component becomes part of the IgA molecule as the immunoglobulin passes through the epithelial or acinar cells of the salivary glands. During this process, the secretory component envelops the Fc region of IgA. Its primary role is to protect the immunoglobulin from the action of various salivary proteases, which are abundant in saliva. Additionally, the secretory component facilitates the transport of secretory IgA to mucosal secretions and helps stabilize the structure of polymeric IgA. It binds to immunoglobulin A via stable covalent disulfide (S-S) bonds. In its molecular form, the secretory component functions as a polyimmunoglobulin receptor and is localized in the basolateral membrane of epithelial (acinar) cells.

The daily production of secretory IgA exceeds that of all other immunoglobulin classes. IgA-secreting plasma cells are densely concentrated along mucosal surfaces. For instance, in the jejunum of the small intestine, there are over 2.5×10^{10} IgA-secreting plasma cells—surpassing the total plasma cell population found in the bone marrow, lymph nodes, and spleen combined. Each day, an individual secretes between 5 g and 15 g of secretory IgA into mucosal secretions.

Plasma cells that synthesize IgA migrate to the subepithelial tissue, where the secreted IgA binds with high affinity to the receptor for polymeric immunoglobulin molecules (Figure 6.6). This “poly-Ig” receptor is expressed on

the basolateral surface of most mucosal epithelia (e.g., lining the digestive, respiratory, and genital tracts) and on glandular epithelia of the mammary, salivary, and lacrimal glands. Upon binding of polymeric IgA to the “poly-Ig” receptor, the receptor-IgA complex is transcytosed across the epithelial barrier into the lumen. This transport process involves receptor-mediated endocytosis at coated pits, followed by directed vesicular trafficking through the epithelial cell to the luminal membrane, where vesicle fusion with the plasma membrane occurs. The poly-Ig receptor is subsequently enzymatically cleaved from the membrane, leaving the secretory component bound to and released together with polymeric IgA into the mucosal secretions. The secretory component shields the protease-sensitive regions of the hinge area of secretory IgA, thereby enabling the polymeric molecule to remain stable for extended periods in the protease-rich mucosal environment, which would otherwise degrade it. Pentameric IgM is also transported into mucosal secretions via this mechanism, although it represents a much smaller proportion of antibodies in mucosal secretions compared to IgA. The poly-Ig receptor binds to the J-chain present in both polymeric IgA and IgM antibodies.

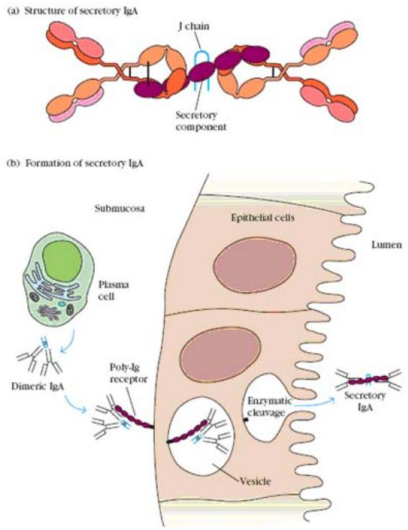


Figure 6.6: *Structure and formation of secretory IgA. (a) Secretory IgA contains two IgA molecules covalently linked to each other by a J-chain as well as the secretory component. The secretory component has five domains similar to those in Ig that are linked to the IgA dimer by a disulfide bond between its fifth domain and one of the IgA heavy chains. (b) Secretory IgA is formed during transport across the mucosal membrane of epithelial cells. The IgA dimer binds to the poly Ig receptor on the basolateral membrane of the epithelial cell and enters the process of endocytosis. After transport, the receptor-Ig complex is enzymatically degraded and the secretory component is released from the IgA dimer.*

The molecular mass of secretory immunoglobulin A is approximately 400,000 Da (comprising two monomers of 160,000 Da each, the J-peptide of 15,600 Da, and the secretory component of 70,000 Da).

Two isotypes of immunoglobulin A exist: IgA_1 and IgA_2. Their distribution and relative abundance vary according to the mucosal tissue location. IgA_1 predominates in the upper respiratory tract and the upper gastrointestinal tract, whereas the concentration of IgA_2 increases in the lower segments of the gastrointestinal tract.

The induction of IgA production occurs via two mechanisms. Antigens enter the oral cavity, stimulating the proliferation and differentiation of B-lymphocytes located in the salivary glands. It is presumed that antigens access the salivary gland ducts through retrograde saliva flow. Subsequently, the antigens are internalized by ductal epithelial cells via endocytosis and transported to the local lymphoid tissue, where they are presented to T and B lymphocytes. Activated B-

lymphocytes differentiate into plasma cells, which synthesize IgA (Figure 6.7). The produced immunoglobulin A, during its passage through the epithelial cells of the acini and ducts, acquires the secretory component and, as a mature secretory immunoglobulin A molecule, is secreted into saliva.

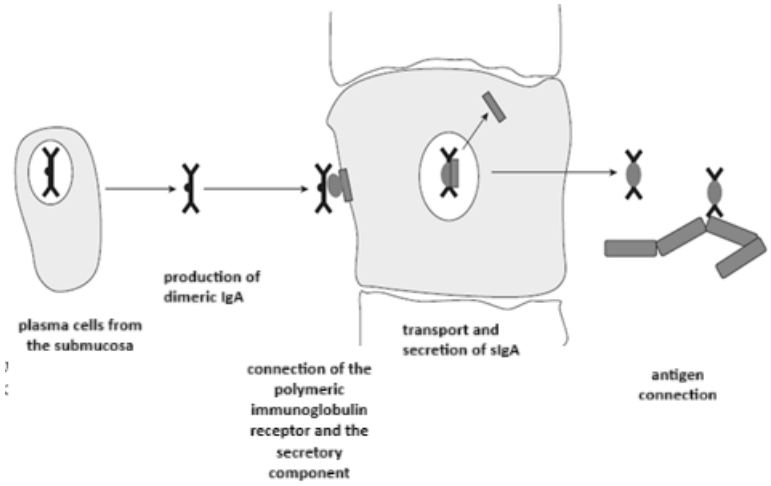


Figure 6.7: *Stages of IgA production*

The second mechanism of IgA production involves the migration of B lymphocytes from the lymphoid tissues of the digestive tract (solitary lymph nodes and Peyer's patches) to the salivary glands. Following antigen stimulation and presentation to lymphocytes, these cells exit the intestinal lymphoid tissue and migrate to the salivary glands. Within the salivary glands, B lymphocytes undergo differentiation and blast transformation into plasma cells, which subsequently produce IgA.

Secretory IgA has several important biological functions for the protection of oral tissues. They are:

1. inhibition of bacterial adherence to oral tissues and prevention of penetration of antigens through the oral mucosa;
2. neutralization of bacterial toxins;
3. neutralization of viruses by blocking their adherence to cell receptors;
4. synergism with other mechanisms of local immune defense of the host;

Secretory IgA exerts a crucial effector function at mucosal surfaces,

which constitute the primary entry points for most pathogenic organisms. Due to its polymeric nature, secretory IgA is capable of cross-linking large antigens containing multiple epitopes. The binding of secretory IgA to bacterial or viral surfaces inhibits the attachment of pathogens to mucosal cells, thereby preventing viral infections and bacterial colonization. The complexes formed between secretory IgA and antigens are efficiently trapped within the mucus and subsequently cleared by ciliated epithelial cells of the respiratory tract or by intestinal peristalsis. Secretory IgA has been demonstrated to serve as a vital line of defense against bacteria including *Salmonella*, *Vibrio cholerae*, and *Neisseria gonorrhoeae*, as well as viruses such as poliovirus, influenza virus, and reovirus.

Breast milk contains secretory IgA along with numerous other molecules that contribute to the protection of the newborn against infections throughout life. Given that the immune system of infants is not yet fully developed, breastfeeding plays a crucial role in safeguarding the health of newborns.

CHAPEROKINES (HSP70/HSPA)

This protein family, also referred to as Heat Shock Proteins (HSP70/HSPA), has a molecular mass of approximately 70 kDa and is found in numerous cells, tissues, and various body fluids. Their presence has also been confirmed in saliva. Within this fluid, two types of chaperones are identified: constitutive (constantly present) chaperones and inducible chaperones that appear in response to specific stimuli. The presence of HSP70/HSPA proteins in the oral environment is linked to the oral defense mechanisms.

The salivary glands represent the primary source of chaperone proteins (HSP70/HSPA) found in saliva. The concentrations of these proteins vary considerably among individuals in both whole saliva and parotid saliva. Unlike other salivary proteins, chaperones are likely not secreted via the classical exocytotic pathway in acinar cells. It is presumed that these proteins reach the oral cavity through passive transport from blood plasma across the acinar and ductal cells of the salivary glands.

Other significant sources of salivary chaperones include mucosal epithelial cells, gingival crevicular fluid, oral mucosal transudate, and gingival bleeding.

Salivary chaperones have been shown to bind and agglutinate both Gram-positive bacteria (such as *Streptococcus mutans* and *Streptococcus mitis*) and Gram-negative bacteria (such as *Escherichia coli*). Due to the capacity of salivary HSP70/HSPA proteins to form dimers and oligomers, their bacterial agglutination ability is enhanced. Furthermore, it has been demonstrated that chaperones also bind to tooth hydroxyapatite, thereby contributing to the formation of the acquired dental pellicle.

Salivary chaperones also participate in the specific immune defense of the oral cavity. They stimulate the release of various proinflammatory cytokines from cells such as monocytes, macrophages, and T-lymphocytes, and promote the production of nitric oxide (NO) by macrophages. Salivary HSP70/HSPA proteins further contribute to immune responses by activating natural killer (NK) cells and initiating complement activation [49–51]. Additionally, these small salivary peptides are involved in antigen presentation to cytotoxic T lymphocytes and NK cells.

HSP70/HSPA proteins have an important immunological role, which

involves opsonization of bacteria, which facilitates the role of polymorphonuclear cells that destroy bacteria. Polymorphonuclear cells (neutrophil leukocytes) constantly arrive in saliva through the gingival fluid. In case of inflammation of the gingiva or certain wounds on the oral mucosa, the number of these defense cells in the gingival fluid increases.

Extracellular HSP70/HSPA proteins present in saliva have a cytoprotective role. This role is based on three different mechanisms:

1. non-specific binding of HSP70/HSPA proteins to epithelial cells, thus protecting the oral mucosa from the penetration of bacterial toxins,
2. specific (achzin type) binding of these proteins to sulfoglycolipid structures of epithelial cells, thus preventing bacterial colonization,
3. reduction of the mechanism of apoptotic death of epithelial cells.

More recently, the synergistic effect of salivary chaperones and histatin-5, which would protect the oral mucosa from candidomycotic infection, has been investigated.

7 ACID-BASE REGULATION, ANTIOXIDANT PROTECTION AND THE IMPORTANCE OF SALIVA

REGULATORS OF SALIVARY pH

Bicarbonate buffer

Phosphate buffer

Protein buffer

ANTIOXIDATIVE PROTECTION OF SALIVA

Oral peroxidase, catalase, superoxide dismutase, glutathione peroxidase, glutathione reductase

Non-enzymatic antioxidants

SALIVARIC ENZYMES AND LIPIDS PRESENT IN SALIVA

Salivary enzymes important for the diagnosis of periodontal diseases

Lipids

IMPORTANCE OF SALIVA IN MAINTAINING ORAL HEALTH

REGULATORS OF SALIVARY pH

The actual acidity (pH) of the oral environment is directly influenced by the presence and quantity of mixed saliva. Despite minor fluctuations, salivary secretion is continuously released into the oral cavity, resulting in a thin film of saliva covering all areas of the oral environment, including the teeth and oral mucosa. This thin, fluid layer of saliva is also present on the mucosa of the hard palate and the teeth of the upper jaw. However, the situation differs slightly in the lower jaw and the mucosa of the floor of the mouth. Due to the anatomical location of the excretory ducts of the two submandibular and two sublingual glands, saliva accumulates abundantly in these areas, making the presence of only a thin salivary film unlikely.

Given the physiological flow of saliva (its transfer to the stomach upon swallowing) and its buffering capacity, mixed saliva plays a key role in determining the pH of the oral environment. Therefore, measuring the acidity of mixed saliva provides an approximate value for the acidity of the oral cavity. Studies measuring the pH of mixed saliva have shown a wide range of values, from highly acidic (pH 5.3) to alkaline (pH 7.8). These variations in pH depend on factors such as the time of measurement and the volume of salivary secretion—specifically, whether the saliva is unstimulated or stimulated.

Buffers regulate the electrochemical reactions occurring in saliva. These systems typically consist of a combination of weak acids (or weak bases) and their corresponding salts, or they may be amorphous compounds that react with strong acids or bases to form weaker, less reactive acids or bases and corresponding salts.

Mixed saliva contains several buffers. It is more appropriate to speak of the buffer mechanism of this secretion. This mechanism consists of the following buffers:

- bicarbonate buffer
- phosphate buffer
- protein buffer
- urea
- salivary amylase as a preventive buffer
- preventive-prophylactic buffer of fluorides

Bicarbonate buffer

The bicarbonate buffer is the predominant buffering system in mixed saliva during stimulated salivary secretion. Biochemically, it consists of sodium bicarbonate ($NaHCO_3$) and carbonic acid (H_2CO_3) in a typical ratio of 1:20. In unstimulated saliva, the concentration of this buffer is approximately 1 mmol/L, whereas in stimulated salivary secretion, it can rise significantly, reaching up to 60 mmol/L. When a strong acid is introduced into the oral cavity, sodium bicarbonate neutralizes it by forming weak carbonic acid, which subsequently dissociates into carbon dioxide (CO_2) and water (H_2O), as shown in the reaction:

$$NaHCO_3 + HCl = NaCl + H_2CO_3$$

$$H_2CO_3 \rightarrow H_2O + CO_2$$

In the presence of a strong base, carbonic acid will react with it and form the corresponding salt:

$$H_2CO_3 + NaOH = NaHCO_3 + H_2O$$

Thus, instead of a strong base, the solution will contain sodium bicarbonate, which has less of an effect on the electrochemical reaction than sodium hydroxide.

The significance of this buffer and its increased concentration is multiple:

1. The pH values reach 7.8, whereby the mixed saliva becomes slightly alkaline.
2. The alkalization of saliva increases the stability of the larger number of proteins and glycoproteins in this secretion, so that these molecules can maximally perform their function in maintaining oral homeostasis.
3. The dissolving property of the main component of saliva - water - is reduced, which protects the solid dental substances from mineral loss.
4. In alkaline conditions, the remineralization of the surface layers of the tooth is stimulated as a result of the deposition of minerals from saliva on the tooth surface. This is actually the first and most important phase of the recrystallization of the solid dental surfaces.
5. Alkaline saliva creates the preconditions for the adsorption of salivary glycoproteins onto the tooth surface during mastication. In this way, the occlusal surfaces of molars and premolars as well as the incisal edges of canines and incisors are protected from excessive wear of the tooth substance.

Phosphate buffer

The phosphate buffer is the predominant buffering system in unstimulated salivary secretion. Its concentration ranges from 7–8 mmol/L under unstimulated conditions and decreases to 2–3 mmol/L during stimulated salivary secretion. This buffer system in saliva consists of primary sodium phosphate (NaH_2PO_4) and secondary sodium phosphate (Na_2HPO_4). Within this system, the primary phosphate acts as a weak acid and reacts with bases to form secondary phosphate and the phosphate salt of the ingested base.

$$NaH_2PO_4 + NaOH = Na_2HPO_4 + H_20$$

Secondary phosphate acts as a base and reacts with acids, producing primary phosphate and the sodium salt of the corresponding acid.

$$Na_2HPO_4 + HCl = NaH_2PO_4 + NaCl$$

The importance of the phosphate buffer during unstimulated salivary secretion is multiple:

1. With its concentration of 7-8 mmol/l, it reduces the pH value of saliva to 6.1, which is why the salivary secretion is slightly acidified.

2. The weak acidity of saliva increases its dissolving capabilities and thus the self-cleaning effects of the oral cavity are satisfied.

3. The increased concentration of the phosphate buffer causes desorption of the absorbed glycoproteins and thus cleans the teeth from the created dental pellicle. Because of this function, the phosphate buffer is called the oral cavity cleaner.

The phosphate buffer plays a key role in the self-cleaning mechanism of the teeth and oral environment during the resting phase between meals. During this phase, the phosphate buffer facilitates the removal of salivary glycoproteins that accumulate on the tooth surface during periods of increased salivation. These glycoproteins are initially deposited to protect the teeth from mechanical abrasion during mastication. Once mastication ends, this protective layer is no longer needed and is subsequently cleared from the tooth surfaces through the action of the phosphate buffer.

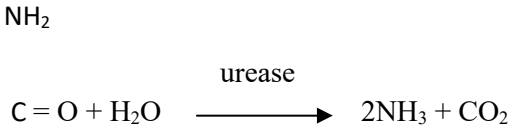
Protein buffer

The buffering capacity of proteins stems from their amphoteric nature (depending on the pH of the environment, they can act either as acids or bases). This characteristic is enabled by the presence of free carboxyl (acidic) and basic groups, particularly amino groups. In an alkaline environment, proteins behave as weak acids, binding alkaline ions such as sodium and potassium. Conversely, during acidosis, they release these alkaline ions to neutralize excess acids. However, due to their relatively low concentration in saliva, proteins play only a minor role in maintaining the electrochemical balance of this fluid.

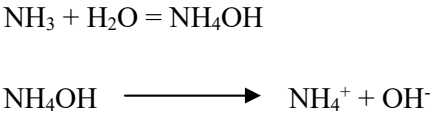
In addition to the aforementioned buffers, other compounds or enzymes that have a specific buffering role also participate in maintaining the electrochemical reaction of the oral environment, and these are:

- urea,
- salivary amylase as a preventive buffer, and
- preventive-prophylactic buffer of fluorides.

Urea is the diamide of carbonic acid. It is not synthesized by the salivary glands but reaches them through ultrafiltration of blood serum at the level of the acinar cells of the salivary glands. As the end product of protein catabolism, urea exhibits moderately alkaline behavior in solution. Due to its low molecular weight, it readily diffuses through various deposits, particularly through the layers of dental plaque. Within plaque, urea is broken down by the enzyme urease according to the following reaction:



The released ammonia reacts with water and forms a strong base NH4OH (ammonium hydroxide) in the plaque, which dissociates into ammonium and a hydroxyl group:



As a result of these processes, the microenvironment within dental plaque becomes alkalized in proportion to the concentration of ammonium hydroxide produced. In this manner, the body counteracts an uncontrolled drop in pH below 5.5 which is the critical threshold at which demineralization of the hydroxyapatite in dental tissues begins. It has been established that urea derived from saliva plays a predominant role in regulating the acid-base balance within dental deposits (dental plaque). Notably, urea is capable of diffusing only through the layers of young, immature plaque. The pH changes within the plaque environment will be addressed in more detail in the section of this book dedicated to dental plaque.

Salivary amylase as a preventive buffer is the most important enzyme in saliva, responsible for hydrolyzing retained starch particles and thereby effectively removing them. This action prevents bacteria from fermenting starch into lactate. Through its amylolytic activity, amylase not only contributes to the self-cleaning function of the oral environment but also exerts a significant preventive buffering effect. In doing so, amylase indirectly inhibits the uncontrolled decline in the pH value of the oral environment.

The preventive effect of fluorides stems from their strong affinity for the enzyme enolase, which plays a key role in glycolysis. By inhibiting enolase, fluorides disrupt glucose catabolism, resulting in reduced production of final metabolites such as lactate. Consequently, this limits the drop in environmental pH and prevents the onset of dental demineralization.

ANTIOXIDATIVE PROTECTION OF SALIVA

Oral peroxidase, catalase, superoxide dismutase, glutathione peroxidase, glutathione reductase

Oral peroxidase, catalase, superoxide dismutase, glutathione peroxidase, and glutathione reductase are salivary enzymes that play a key role in antioxidant defense. In contemporary research, the development of various diseases (including those affecting the oral cavity) is increasingly associated with the harmful impact of free radicals. These are atoms, ions, or molecules that possess one or more unpaired electrons in their structure. Due to the presence of the latter, free radicals are strongly reactive and can interact with various biomolecules such as proteins, lipids, and lipoproteins, leading to oxidative damage that compromises their structure and function.

In this manner, free radicals can cause damage to various biological structures, including blood vessel walls, cell membranes, and DNA. Under physiological conditions, free radicals are naturally generated in the human body as a byproduct of cellular processes. These processes include oxidative phosphorylation in mitochondria, phagocytosis, the biotransformation of substances in the endoplasmic reticulum, ethanol metabolism, and oxidative reactions involving metals.

The most well-known free radicals are byproducts of oxygen metabolism and include the superoxide anion (O_2), perhydroxyl radical (HOO), hydrogen peroxide (H_2O_2), hydroxyl radical (OH), among others. The body combats these reactive species through specialized defense mechanisms collectively known as antioxidant protection. This protection involves both organic and inorganic molecules capable of neutralizing free radicals. These molecules are classified into enzymatic and non-enzymatic antioxidants. Among the enzymatic antioxidants, the most significant are oral peroxidase, catalase, superoxide dismutase, glutathione peroxidase, and glutathione reductase. These enzymes constitute the first line of defense in the body's antioxidant system.

Oral peroxidase is secreted by the major salivary glands, predominantly by the parotid gland. This enzyme contains selenium at its active site and plays a crucial role in reducing hydrogen peroxide (H_2O_2), a metabolic byproduct of oral bacteria. The reduction process occurs in the presence of thiocyanate ions (SCN^-), which act as electron donors—functionally similar to how reduced glutathione operates in other biological systems. Thiocyanates, naturally present in saliva, are derived from hydrogen cyanide (HCN), a component of tobacco smoke. After detoxification of HCN in the liver, thiocyanates are transported via the bloodstream to the salivary glands and subsequently reach the saliva through ultrafiltration.

Salivary peroxidase catalyzes the reaction between hydrogen peroxide (H_2O_2) and thiocyanate ions (SCN^-), resulting in the formation of hypothiocyanic acid (HOSCN) and hypothiocyanate ions ($OSCN^-$). These reaction products exhibit antibacterial activity, which is based on their interaction with the sulfhydryl (SH) groups of bacterial enzymes involved in glycolysis, such as hexokinase, aldolase, and pyruvate kinase. In addition to its role in non-specific antibacterial defense within the oral environment, salivary peroxidase also plays a key role in the effective elimination of hydrogen peroxide, a potential free radical from the oral cavity.

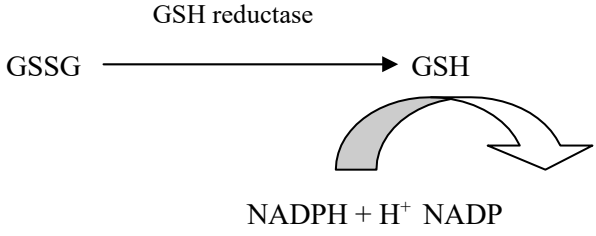
Superoxide dismutase (SOD) catalyzes the dismutation reaction of oxygen (O_2) to hydrogen peroxide. In prokaryotic organisms, superoxide dismutases containing Fe^{2+} and Mn^{2+} are present. Eukaryotic organisms contain two forms of SOD: a cytosolic dimer (Cu, Zn-SOD) and a tetramer (Mn-SOD), localized in mitochondria.

$$O_2 + O_2 + 2H^+ \xrightarrow{\text{SOD}} H_2O_2 + O_2$$

Glutathione peroxidase (GSH peroxidase) is a tetrameric enzyme, with each subunit containing one selenium atom. This enzyme has a primary role in neutralizing hydrogen peroxide and inhibiting the H_2O_2-dependent mechanism of free radical generation. Glutathione peroxidase utilizes reduced glutathione (GSH) as an electron donor in this reaction, resulting in the formation of oxidized glutathione (GSSG) and water.

$$H_2O_2 + 2GSH \xrightarrow{\text{GSH peroxidase}} 2H_2O_2 + GSSG$$

For continuous reduction of hydrogen peroxide to occur, continuous regeneration of GSSH to GSH is required. This reaction is catalyzed by NADPH-dependent glutathione reductase (GSSG reductase).



Catalase is a tetramer in structure, and each of its subunits contains a HEM that forms part of the enzyme's active site. In the oral cavity, catalase enters saliva from surrounding cells. Within these cells, it is predominantly located in peroxisomes, where it plays a key role in limiting the accumulation of hydrogen peroxide (H_2O_2) under physiological conditions. Catalase catalyzes the breakdown of hydrogen peroxide into molecular oxygen and water.

$$H_2O_2 + H_2O_2 \xrightarrow{\text{catalase}} O_2 + 2H_2O$$

The activity of this enzyme increases with elevated production of hydrogen peroxide (H_2O_2), as it plays an active role in its degradation. It has been observed that the activity of catalase, as well as superoxide dismutase, is increased in patients with recurrent aphthous stomatitis.

Non-enzymatic antioxidants

The primary non-enzymatic antioxidants present in saliva include uric acid, albumin, vitamin C, and glutathione.

Uric acid (*acidum uricum*) represents the final product of purine nucleotide metabolism. Due to the absence of the enzyme uricase in the human body, which prevents the further breakdown of uric acid, and its subsequent reabsorption at the level of the renal tubules, a sufficiently elevated concentration of this compound is maintained in blood plasma. The exact mechanism of its presence in saliva remains insufficiently elucidated. It is postulated that uric acid salts (urates) are transferred from blood plasma into saliva via passive diffusion. The concentration of uric acid in saliva is lower than that in blood plasma. It is also plausible that uric acid reaches the saliva through the gingival crevicular fluid. Irrespective of its origin, uric acid constitutes the principal antioxidant in saliva, accounting for approximately 70% of its total antioxidant capacity.

Uric acid reduces and neutralizes free radicals and is itself oxidized to allantoin. Additionally, uric acid plays a significant role in forming complexes with metal ions, such as Fe^{3+}. In this manner, it decreases the oxidative potential of Fe^{3+} without undergoing oxidation.

In patients with periodontitis and those with oral cancer, a reduced concentration of uric acid in saliva has been observed. This decrease is attributed to the increased "consumption" of this antioxidant in the process of neutralizing free radicals, whose concentrations rise markedly under these pathological conditions.

Albumins are plasma proteins synthesized in the liver. Their functions include maintaining colloidosmotic pressure, regulating blood plasma pH, and facilitating the transport of various substances, while also exhibiting antioxidant activity. They enter the saliva from the bloodstream through the gingival fluid by transudation.

Glutathione is a tripeptide composed of the amino acids glutamic acid, cysteine, and glycine. It exists within cells in two forms: reduced (GSH) and oxidized (GSSG). For proper cellular function, the presence of the reduced form

is particularly critical, as it is essential for the activity of the antioxidant enzyme glutathione peroxidase.

Numerous studies have demonstrated that smoking leads to a reduction in the concentration of glutathione in saliva. Consequently, cigarette smoking significantly diminishes the antioxidant capacity of saliva, affecting both glutathione levels and the activity of the enzyme oral peroxidase. This represents an additional predisposing factor for the development of oral cancer.

SALIVARY ENZYMES AND LIPIDS PRESENT IN SALIVA

Salivary enzymes important for the diagnosis of periodontal diseases

CK (creatine kinase), LDH (lactate dehydrogenase), AST (aspartate aminotransferase), ALT (alanine aminotransferase), GGT (gamma-glutamyltransferase), ACP (acid phosphatase), ALP (alkaline phosphatase) CK (creatine kinase), LDH (lactate dehydrogenase), AST (aspartate aminotransferase), ALT (alanine aminotransferase), and GGT (gamma-glutamyltransferase) are intracellular enzymes that play a crucial role in cellular metabolic processes. They are particularly important for the metabolic activity of cells in the soft oral tissues, such as the gingiva and the oral mucosa. When cellular damage occurs, the concentration of these intracellular enzymes in various body fluids increases. These enzymes are detectable in gingival fluid and saliva, with their salivary concentration rising significantly during inflammatory conditions of the gingiva or periodontium.

ALP (alkaline phosphatase) and ACP (acid phosphatase) are intracellular enzymes found in numerous tissues and organs, with a particular abundance in bone tissue. These enzymes are present in saliva and derive from the gingival fluid. Their concentration in saliva increases during inflammatory processes of the periodontium that are associated with alveolar bone damage.

Lipids

In addition to the primary organic components, proteins and glycoproteins, various lipids have also been identified in saliva. The concentration of total lipids in stimulated parotid saliva is 0.21 mg/100 ml, in submandibular saliva 0.91 mg/100 ml, and in mixed saliva 1.36 mg/100 ml. Among the nonpolar lipids present in saliva are cholesteryl esters, cholesterol, triglycerides, diglycerides, monoglycerides, and free fatty acids. Their

concentrations in the different types of salivary secretions are presented in Table 7.1.

Table 7.1: Nonpolar lipids in saliva originating from different salivary glands and their concentrations (mg/100 ml)

	Parotid saliva	**Submandibular saliva**	**Mixed saliva**
Cholesterols	0.03	0.26	0.43
Triglycerides	0	0.19	0.29
Diglycerides	0	0.05	0.26
Free fatty acids	0.02	0.07	0.10
Cholesterol	0.09	0.17	0.13
Monoglycerides	0.07	0.15	0.10
Total lipids	**0.21**	**0.89**	**1.31**

Polar lipids present in salivary secretions are: phosphatidylcholine, phosphatidylethanolamine and sulfatides (table 7.2).

Table 7.2: Percentage of individual types of polar lipids in saliva relative to total polar lipids (%)

	Parotid saliva	**Submandibular saliva**	**Mixed saliva**
Phosphatidylcholine	14	9	1
Phosphatyl-inositol	15	23	9
Sulfatides	8	28	7
Unidentified lipids	63	40	78

The predominant lipids in saliva are nonpolar lipids. Among the lipids isolated from parotid saliva, 99% are nonpolar lipids. Submandibular saliva similarly contains a high concentration of nonpolar lipids (approximately 98%), while in mixed saliva, nonpolar lipids constitute 96.4%. Ultracentrifugation of saliva does not allow for the separation of individual lipid types, as is feasible

with blood plasma lipids. The origin of lipids in saliva, as well as their physiological role within this secretion, remains undetermined. Given that these are nonpolar molecules insoluble in water, their persistence in saliva raises questions. It has been established that salivary lipids do not form complex lipoprotein particles. It is hypothesized that lipids in saliva form specific "aggregates" with salivary proteins and glycoproteins, the nature and function of which have yet to be elucidated.

The presence of lysophosphatidic acid has also been identified in saliva. This organic compound serves as a multifunctional mediator in numerous biological processes. The concentration of lysophosphatidic acid in saliva is approximately 0.785 μmol/ml. Its primary role in saliva involves the facilitation of wound healing in the mucous membranes of the upper gastrointestinal tract.

IMPORTANCE OF SALIVA IN MAINTAINING ORAL HEALTH

The principal role and significance of saliva lie in maintaining oral homeostasis. The term homeostasis encompasses all mechanisms that contribute to preserving the constant biochemical composition of the organism's internal environment. Oral homeostasis specifically refers to the collection of mechanisms involved in sustaining and protecting the health of all oral structures; in simpler terms, it pertains to the preservation of the health of the teeth and oral mucosa.

A tool that enables oral homeostasis is saliva, that is, all its organic and inorganic components. We will list all the roles of saliva in maintaining oral homeostasis:

1. Dissolution and decomposition of residual food particles and their removal from the oral cavity (self-cleaning of the oral cavity).

2. Role of saliva in chewing food, speaking and swallowing.

3. Protective role, which can be divided into:

- antibacterial protection - aggregating, bactericidal and bacteriostatic action,
- antiviral protection,
- antifungal protection,
- protection of tooth enamel from attrition,
- protection from chemical agents,

- buffering role (prevention of major variations in the pH of the environment),
- protection from demineralization of tooth enamel

4. Role of saliva in water metabolism.

5. Excretory role.

6. Role of saliva in maintaining the stability of prosthodontic restorations in the oral cavity.

7. Role of saliva in the sense of taste.

8. Antioxidant protection.

9. Anticancer effect.

10. Biological material.

The self-cleaning function of the oral cavity is facilitated by the primary constituent of saliva—water—and by salivary amylase. Water, acting as a universal solvent, dissolves residual food particles, which are then removed from the oral cavity through swallowing. However, these effects of saliva pertain only to particles that are soluble in water, i.e., water-soluble food particles. Components insoluble in water, such as starch—which constitutes approximately 45% of the daily diet—are initially broken down by salivary amylase into products that are readily soluble.

Antibacterial, antiviral, and fungicidal protection in saliva is mediated through mechanisms of both specific and non-specific immunity. The specific immune protection consists of secretory immunoglobulins present in saliva: IgA, IgG, and IgM. The non-specific protective components of saliva include lysozyme, lactoferrin, transferrin, the salivary peroxidase system (comprising peroxidase enzymes, hydrogen peroxide, thiocyanates, and hypothiocyanates), proline-rich proteins, histatins, cystatins, fibronectin, defensins, calprotectins, and cathelicidins.

Protection of tooth enamel from attrition is provided by glycoproteins present in saliva, which form an organic coating on the enamel surface known as the acquired dental pellicle. This dental pellicle exerts a lubricating effect, thereby partially mitigating enamel loss during mastication.

The buffering function of saliva is mediated by multiple salivary buffers that maintain the pH of the oral environment within physiological ranges (6.1–7.8). The primary and most significant buffers in saliva are the bicarbonate

and phosphate systems. The protein buffer plays a less prominent role due to the relatively low concentration of proteins in saliva.

Saliva is also important for protecting tooth enamel from the undesired process of demineralization. It facilitates the opposite process, termed **remineralization**, which restores the damaged surface layers of tooth enamel. Among the components of saliva involved in remineralization, calcium ions and phosphate ions are the most significant. Salivary secretion is saturated with these ions, which constitute the primary components of the hydroxyapatite crystals in tooth enamel.

Saliva also plays a crucial role in **regulating water metabolism** within the body. In cases of significant water deficit (dehydration), saliva secretion is markedly reduced. This mechanism contributes to the conservation of water in the human organism.

During the process of salivation, **certain substances are eliminated from the body,** including final metabolites such as urea, uric acid, and bilirubin. However, this route of excretion is less significant compared to the elimination of these metabolites via the kidneys or the digestive tract.

By dissolving nutrients within the oral cavity, **saliva facilitates the sense of taste**. Nutrients must be dissolved to interact effectively with the taste receptors located on the dorsal (upper) surface of the tongue.

In recent years, growing attention has been directed towards the **antioxidant protection by saliva**. The following salivary enzymes contribute to this vital function: oral peroxidase, catalase, superoxide dismutase, glutathione peroxidase, and glutathione reductase. These enzymes constitute the first line of defense against the detrimental effects of free radicals. The second line of defense involves uric acid, present in saliva and derived from blood plasma.

Individual components exhibit anticancer effects by inactivating numerous mutagenic and **carcinogenic** agents. As the term implies, these harmful substances contribute to the development of various malignant diseases. Saliva possesses the capacity to neutralize many carcinogenic compounds introduced into the body from polluted environments, such as α-toxin, benzopyrene, and tryptophan pyrolysate-1. Inactivation occurs through multiple mechanisms, among which peroxidation of these harmful substances is a key mode of neutralization. Sialoperoxidase, present in saliva, plays a significant role in mediating the anticancer effects of saliva.

Saliva is also important as a **biological material** for the development of

new diagnostic tests aimed at diagnosing and elucidating the pathogenesis of numerous oral and systemic diseases. This is particularly relevant for diseases affecting the tissues of the oral cavity, which are in direct contact with saliva. Consequently, saliva can serve as a “mirror” of oral health. Diseases of the oral tissues are of special interest due to the increasing prevalence of periodontitis and oral cancer.

Saliva analysis is not only important for dental diseases but is also gaining increasing significance in the broader field of medicine. Modern medical practice predominantly relies on blood sample analysis, utilizing increasingly sophisticated techniques and advanced laboratory tools. However, blood sampling is an invasive procedure, which limits its applicability, particularly in populations such as children, the elderly, disabled individuals, and patients requiring continuous clinical-biochemical, pharmacological, or toxicological monitoring. In these circumstances, identifying an alternative body fluid that can be collected more easily and that reflects comparable clinical-biochemical, pharmacological, and toxicological parameters to blood is of great importance. Saliva meets these criteria in certain cases and represents a viable alternative to blood. Recent research has explored the potential of saliva as a diagnostic fluid. Literature data indicate that numerous components measurable in blood, including hormones, medications and their metabolites, drugs and their metabolites, as well as various toxic substances, can also be detected in saliva.

Table 7.3: The most important functions of saliva

Protection of the oral mucosa	It coats the oral mucosa and protects it from mechanical, thermal and chemical irritations. It facilitates speech, chewing and swallowing.
Tooth enamel protection	It is saturated with electrolytes that accelerate mineralization (repair) of damaged tooth enamel.
Buffer capacity	Saliva buffers prevent unphysiological pH changes in the mouth and thus prevent tooth demineralization.
Self-cleaning of the oral cavity	With the help of water and salivary amylase, it contributes to the removal of leftover food particles in the mouth.
Antimicrobial effect	The mechanisms of specific and nonspecific protection enable the control of the oral microflora.
Protecting tooth enamel from attrition	Various salivary glycoproteins, by creating a dental pellicle, partially reduce the wear of tooth enamel during mastication.
Role in the sense of taste	Saliva enables the functioning of the sense of taste because it acts as a solvent for various nutrients.
Excretory role	Saliva enables the elimination of certain harmful substances in the body.
Water homeostasis in the body	In conditions of dehydration, saliva secretion is significantly reduced.

8

BIOCHEMICAL CHARACTERISTICS OF ORAL CONNECTIVE TISSUE STRUCTURES AND BIOLOGICAL MINERALIZATION

COLLAGEN

Elastic and oxytalan fiber system

Glycosaminoglycans

ALVEOLAR BONE, TEETH AND PERIODONTIUM

BIOCHEMICAL CHARACTERISTICS OF FIBRILLAR COLLAGEN

Biochemical characteristics of collagen synthesis

BIOLOGICAL MINERALIZATION

Structure of the alveolar bone

How does biological mineralization occur?

Biochemical composition and structure of alveolar bone

Alveolar bone remodeling

Mechanism of calcification of alveolar bone

Mechanism of bone breakdown (resorption)

Bone remodeling processes

Connective tissue (stroma) constitutes the internal solid framework of the organism, comprising cells embedded within a matrix. The principal components of connective tissue are collagen fibers, which are embedded in a polysaccharide matrix composed of hyaluronan and various glycosaminoglycans. Stromal cells derive from mesodermal embryonic cells that differentiate into fibroblasts, chondroblasts, and osteoblasts, responsible for forming connective tissue, cartilage, and bone, respectively.

The stromal matrix is covered by epithelium of ectodermal origin. At the interface between connective tissue and epithelium, fibroblasts interact with epithelial cells to form the basal lamina (basement membrane). The basement membrane functions as a semipermeable barrier, separating specialized cells, such as epithelial or muscle cells, from soluble stromal proteins. A dense capillary network located immediately beneath the epithelium ensures the supply of nutrients and low molecular weight molecules, such as chemokines, to the epithelial cells. The basement membrane restricts the passage of soluble plasma proteins into the stromal fluid and, conversely, prevents stromal proteins from entering the epithelial fluid.

COLLAGEN

Collagen is a glycoprotein that constitutes approximately 25% of the total protein content in the human body. It is a rigid, water-insoluble molecule characterized by high tensile strength. The structural organization of collagen molecules varies according to their functional roles within specific tissues.

Collagen is the principal protein of connective tissue. Two major genetically encoded groups of collagen exist: fibrillar and non-fibrillar. Collagen fibers, the most abundant fiber type, are visible to the naked eye. Collagen fibrils can be observed using a light microscope, whereas collagen microfibrils (filaments) require electron microscopy for visualization. Beyond their role in forming and maintaining tissue integrity and stability, collagen fibers create a bioactive surface that regulates cell differentiation, morphogenesis, and cell migration (motility). Collagen also plays a critical role in wound healing and various inflammatory processes.

According to the type of polypeptide chains, several types of collagen are distinguished: I, II, III, V, XI. Type I collagen is the most abundant, but a combination of different types of collagen is often found in connective tissues. Type I collagen fibers are often found in combination with type V collagen fibers for several different reasons:

- to facilitate corneal transparency,

- to limit the thickness of the fibers during tissue repair and to help form the architecture of various collagen-containing tissues, such as tendons or the placenta.

Type II collagen fibers, which are unique to cartilage, form a complex with type XI collagen fibers to limit thickness and enhance binding to glycosaminoglycans. Reticular fibers are composed primarily of type III collagen and are extensively covered with glycosaminoglycans and glycoproteins. In the dermis of the skin and in the gingiva, reticular fibers extend beyond the type I collagen fibers, which are already associated with type V collagen. In bone marrow and other less fibrous tissues, type III collagen is associated with a mixture of nonfibrillar types VIII and X.

All collagen fibers, fibrils, and microfibers have an alternating, light and dark colored appearance (cross-striped, ribbon-like). This appearance is due to the extended sequence of tropocollagen triple helices.

Elastic and oxytalan fiber system

Elastic fibers may also be present in the connective tissue of oral tissues, conferring elasticity to the connective tissue. These fibers consist primarily of the protein fibrillin, with or without a central core of elastin protein. Elastic fibers are especially abundant in ligaments and large arteries. Oxytalan fibers, which are bundles of fibrillin fibers lacking elastin, are also found in the periodontium (Figure 8.1).

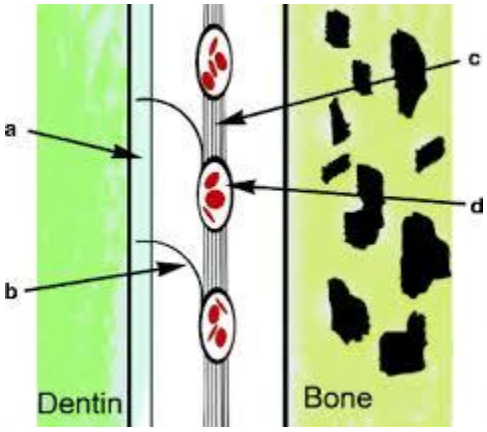
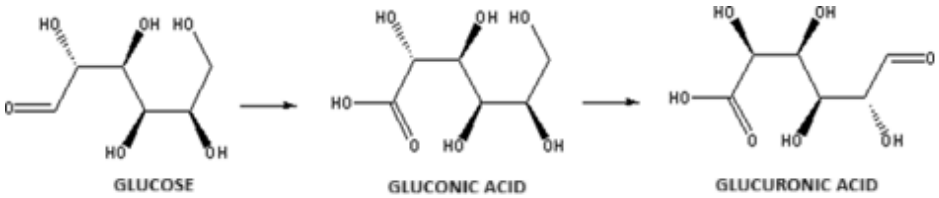


Figure 8.1: a. *cementum* ***b.*** *primary oxytalan fibers* ***c.*** *oxytalan fiber bundle* ***d.*** *periodontal blood vessels. The oxytalan fibers form a network that connects blood vessels and cementum.*

Glucosaminoglycans

Glycosaminoglycans are carbohydrates essential for the development and repair (healing) of connective tissue and for cartilage formation. During tissue development or following damage caused by infection or injury, fibroblasts become activated and proliferate. These cells secrete hyaluronan, a large repeating disaccharide composed of glucuronic acid linked to N-acetylglucosamine (Figure 8.2). Fibroblasts then migrate into the hyaluronan matrix along with proliferating endothelial cells that form new capillaries. Within the hyaluronan, fibroblasts secrete collagen fibers, thereby increasing the density of the stroma. Proteoglycans are proteins covalently bound to glycosaminoglycans. They are synthesized by fibroblasts, chondroblasts, and osteoblasts alongside collagen. These negatively charged polymers confer resilience (elasticity) to connective tissue and are especially important in cartilage. The stroma, or connective tissue, exhibits both resilience and

fibrousness. The long hyaluronic acid molecules are particularly crucial for maintaining the viscosity of fluid in joints surrounding cartilage between bones, as well as within the ocular cavities.



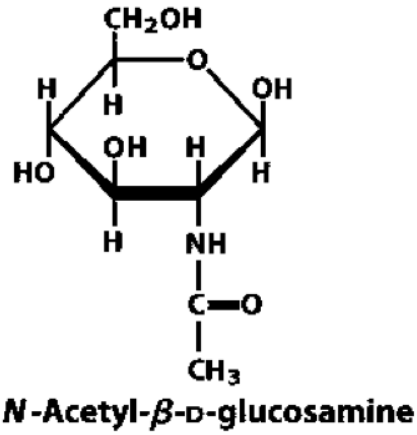


***Figure 8.2**: Chemical structure of glucuronic acid and β-D-N-acetylglucosamine*

ALVEOLAR BONE, TEETH AND PERIODONTIUM

Bone is formed by cells known as osteoblasts. These cells initially secrete collagen, predominantly type I collagen, which constitutes the uncalcified osteoid matrix. Osteoblasts subsequently facilitate the transport of calcium ions from the bloodstream into the osteoid matrix. During the process of calcification, monocytes migrate from adjacent blood capillaries and adhere to irregularities on the calcifying bone surfaces, where they differentiate into osteoclasts. Osteoclasts are specialized cells responsible for bone resorption. Their development is influenced by genetic and environmental factors. These cells play a critical role in shaping bone, particularly in response to mechanical pressure applied to bone tissue.

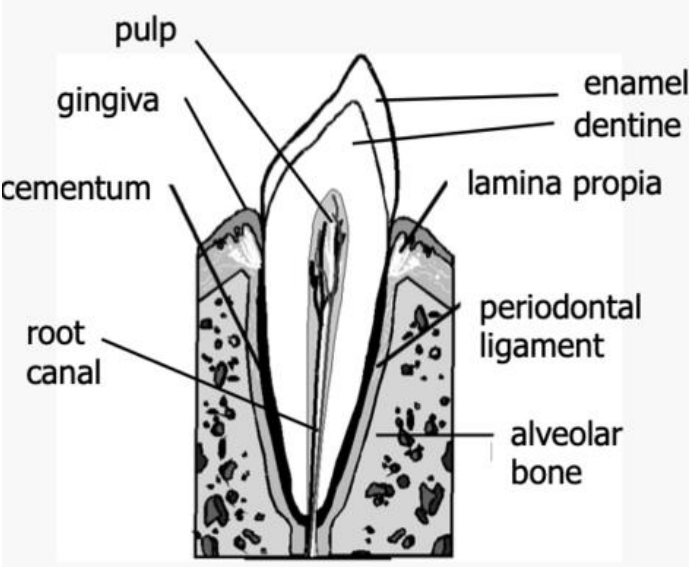


Figure 8.3: *Schematic representation of a tooth with surrounding periodontal tissues*

The tooth and its surrounding periodontal structures are depicted in Figure 8.3. Enamel is produced by ameloblasts, which are cells of ectodermal origin. Enamel is the only calcified tissue in vertebrates that lacks collagen. It forms a hard, impermeable shell encasing the dentin. Type I collagen constitutes the organic framework of dentin, cementum, and bone. The collagen within dentin is synthesized by odontoblasts, whereas the collagen forming the organic matrix of cementum is produced by cementoblasts.

Upon completion of dentin synthesis, odontoblasts remain viable within the dentin, specifically in the pulp, which is non-calcified tissue. On the outer surface of the dentin, apical to the enamel, fibroblasts differentiate into cementoblasts and initiate the formation of cementum.

The periodontium comprises the gingiva, cementum, periodontal ligament, and alveolar bone. The gingiva and periodontium primarily consist of large bundles of collagen fibers that absorb masticatory forces. The periodontal collagen fibers are termed the periodontal ligament because both ends of these fibers are mineralized, one anchoring in the cementum and the other in the alveolar bone, resembling the ligaments that connect bones. Surrounding the collagen fibers and blood vessels within the periodontium are oxythalan fibers composed of fibrillin. This protein imparts a degree of elasticity necessary for tooth movement (tooth mobility).

The gingiva is located coronally to the periodontal ligament and is divided into attached and free (non-attached) parts. The free gingiva forms the soft tissue wall of the gingival sulcus. It extends from its coronal edge, known as the gingival margin, to the enamel-cement junction. The outer (oral) surface of the free gingiva is covered by keratinized epithelium, whereas its inner (sulcular) surface, adjacent to the tooth enamel, is lined by non-keratinized epithelium (Figure 8.4).

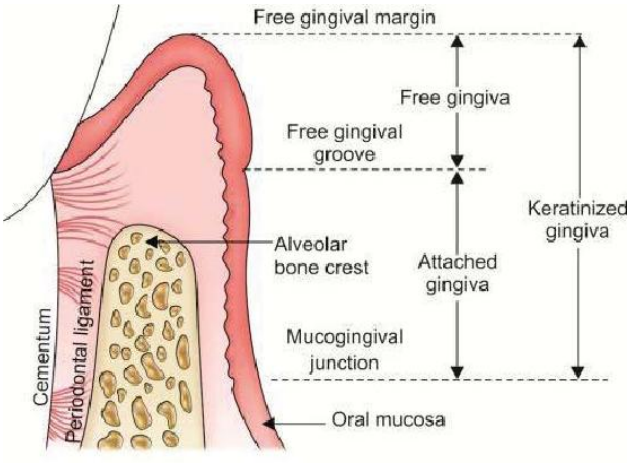


Figure 8.4: *Schematic representation of gingival tissue*

Five groups of collagen fibers are present in the connective tissue of the gingiva, categorized according to their orientation and function. These gingival fibers include: a) dentogingival; b) alveologingival; c) circumferential; d) transseptal; and e) periosteal fibers (Figure 8.5).

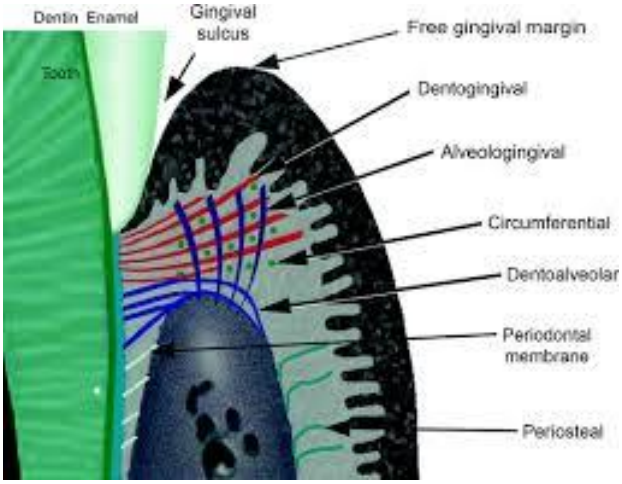


Figure 8.5: *Schematic representation of gingival collagen fibers.*

The first two groups are free gingival fibers, attached at one end to the cementum or bone where the fibers are mineralized, while the other end remains free. These fibers secure the free gingiva firmly against the tooth surface. Circumferential gingival fibers are attached to the cementum and partially encircle the tooth beneath the free gingiva. Transseptal fibers connect the cementum of two adjacent teeth. Periosteal fibers anchor the attached gingiva firmly to the outer surface of the alveolar bone.

Periodontal fibers (periodontal ligament) consist of fundamental bundles of collagen fibers. These fibers are classified according to their location and orientation as follows: A) alveolar crest fibers, B) horizontal fibers, C) oblique fibers, D) apical fibers, and E) interradicular fibers (found in multi-rooted teeth). The portions of the periodontal collagen fibers that insert into the cementum and alveolar bone are termed Sharpey's fibers.

The ground substance, in which the primary periodontal fibers are embedded, consists of proteoglycans that contain embedded oxythalan fibers. These fibers secure and protect the blood vessels within the periodontium during mastication. Biting exerts pressure that compresses the blood vessels in the periodontium. The oxythalan fibers enable the capillaries to retract, allowing immediate restoration of blood flow, including oxygen and essential nutrients, once the pressure is relieved.

BIOCHEMICAL CHARACTERISTICS OF FIBRILAR COLLAGEN

Collagen fibers exhibit a unique and characteristic amino acid composition (Table 8.1). The most abundant amino acids are glycine, constituting approximately one-third of all amino acids, followed by proline at 23%, alanine at 12%, and others including hydroxyproline, serine, lysine, and hydroxylysine. Among the 20 standard amino acids, tryptophan and cysteine are absent, while the remaining 13 amino acids are present only in minor amounts. As tryptophan is an essential amino acid, a diet composed solely of collagen cannot sustain life in humans and other mammals; thus, collagen has no nutritional biological value.

Table 8.1: Amino acid composition of the α1 chain in tropocollagen

The most important amino acids	Percentage representation	Functions
Glycine	33	Strengthens the van der Walls and hydrogen bonds that connect the three polypeptide chains in the collagen helix
Proline	14	The proline responsible for the extension of the helix
Hydroxyproline	9	The hydroxyl group stabilizes the extended helix at high temperatures
Alanine	12	A small side chain that allows polypeptides to lie lengthwise next to each other and form fibers
Serine	4	Small side chain (similar effects to alanine)
Lysine	3	Responsible for covalent cross-links
Hydroxylysine	1	It enables the incorporation of carbohydrate components into the collagen molecule, as lysine is involved in covalent cross-links.

Collagen fibers consist of α1-, α2-, β-, and γ-polypeptide chains, which together form the basic structural unit of collagen known as tropocollagen. The smallest polypeptides, α1- and α2-, each have a molecular mass of approximately 100 kDa; the α1-polypeptide is slightly larger and therefore exhibits slower migration during polyacrylamide gel electrophoresis.

Analysis of the α1- and α2-polypeptides reveals the presence of glycine at every third residue, following a Gly-X-Y repeating sequence, with proline frequently occupying the X or Y positions (Figure 8.6).

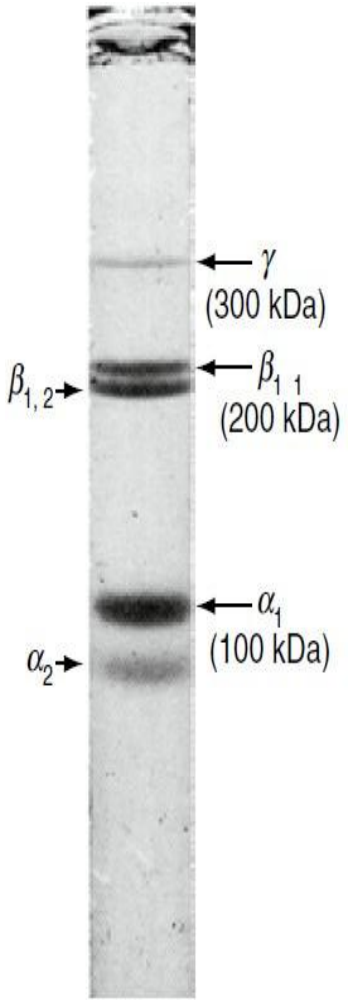


Figure 8.6: *Polypeptide composition of collagen fiber*

It has been confirmed that polypeptides containing the Gly-X-Pro repeat sequence adopt an extended conformation due to the structural properties of the proline peptide bond (Figure 8.7).

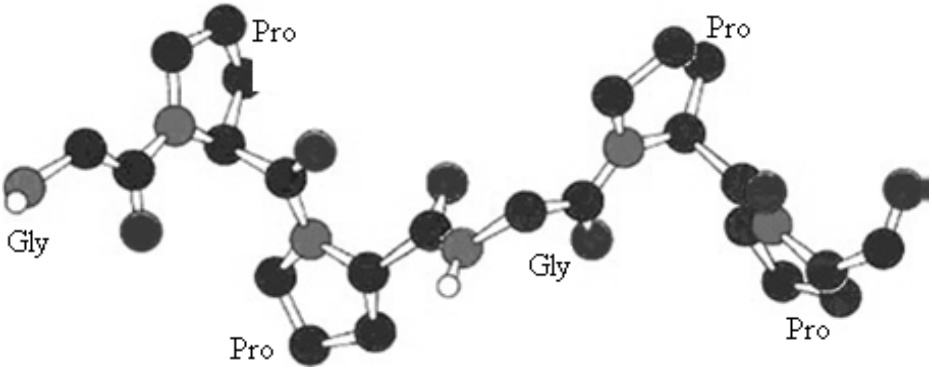


Figure 8.7: *Collagen helix. The secondary structure of collagen is made possible by peptide bonds formed between glycine and proline.*

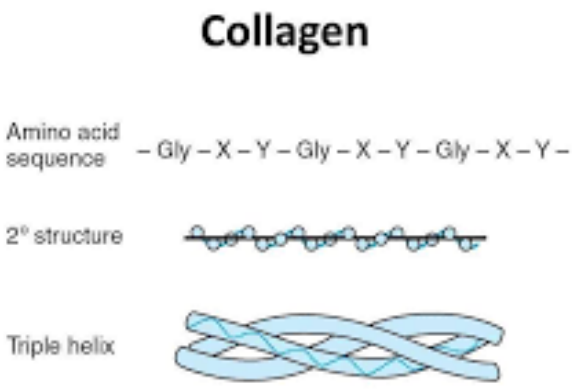


Figure 8.8: *Primary, secondary and tertiary structure of collagen*

Glycine residues facilitate strong linkages between polypeptide chains by enabling the formation of numerous hydrogen bonds within the tropocollagen structure. The amino acid hydroxyproline contributes to the stabilization of tropocollagen, particularly upon exposure to heat.

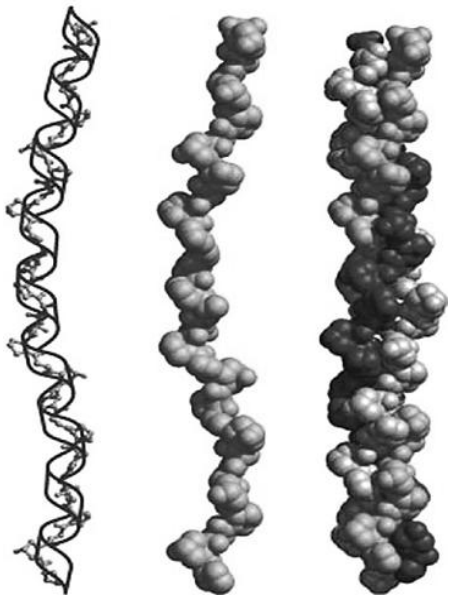


Figure 8.9: *Triple helix of tropocollagen*

Owing to the aforementioned characteristics, namely, the hydrogen bonding capacity of glycine and the roles of proline and hydroxyproline, the polypeptides constituting tropocollagen assemble into an extended triple helix (Figure 8.9). Tropocollagen serves as the monomeric unit of all fibrillar collagens. Two α1- and one α2-polypeptide chains associate through their hydrophobic domains, initiating the formation of the triple helical structure.

The folding of the three α-polypeptide chains into a repeating triple helix imparts collagen fibers with their characteristic strength and insolubility. Covalent cross-links between the α-polypeptides further reinforce and stabilize these fibers.

All collagen proteins are composed of procollagen monomer units, each formed by three polypeptide chains arranged in a triple helix. Each collagen type consists of identical or distinct polypeptide chains that are genetically encoded (Figure 8.10). For example, the triple helix of type II collagen is composed of three identical α1 chains. In contrast, type I collagen contains a triple helix formed by two different polypeptide chains (α1 and α2). Type IV collagen comprises three different polypeptide chains. Thus, the varying polypeptide compositions of different collagen types give rise to distinct structural configurations.

The majority of periodontal fibers are composed of type I collagen. The periodontium also contains a substantial proportion of type III collagen (approximately 20%), which is composed of three α chains and includes hydroxyproline, hydroxylysine, and cysteine in its structure. Type III collagen is more fibrillar and elastic than type I and plays a crucial role in maintaining the structural integrity of the periodontal ligament during the horizontal and vertical tooth movements that occur during mastication.

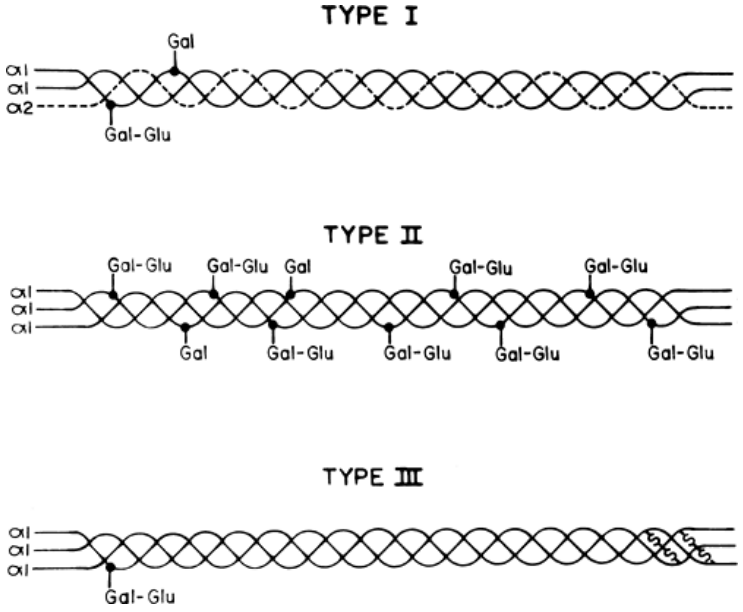


Figure 8.10: *Three types of collagen*

Collagen, based on its biochemical properties, is classified as a glycoprotein because, in addition to its amino acid composition, it contains a carbohydrate component. The protein portion, previously discussed, is predominant in collagen. The carbohydrate component is present as short side chains attached to the main polypeptide chain. These side chains consist of two sugars: glucose and galactose. The attachment of the carbohydrate side chains to the tropocollagen molecule is facilitated by the presence of the amino acids hydroxylysine and hydroxyproline, as the sugar moieties are linked to the polypeptide chain via the hydroxyl (OH) groups of these residues.

Biochemical characteristics of collagen synthesis

Collagen synthesis takes place in the dentin cells-odontoblasts, the alveolar bone-osteoblasts, the cementum-cementoblasts and the oral mucosa-fibroblasts. The collagen synthesis process begins in the aforementioned cells, and the process itself takes place intracellularly in four phases (Figure 8.11):

1. Synthesis of polypeptide chains, which takes place on the ribosomes and endoplasmic reticulum in the cell. **Preprocollagen** is created in this phase.

2. Introduction of hydroxyl groups (OH) to the amino acids lysine and proline (hydroxylation). The amino acids hydroxylysine and hydroxyproline are created, and this hydroxylation process takes place under the action of the *hydroxylase* enzymes. The hydroxylation activity is enabled by the presence of vitamin C.

3. Introduction of glucose and galactose, in the form of side chains (glycosylation). Glucose and galactose are bound to the OH group of the amino acids hydroxylysine and hydroxyproline. The aforementioned glycosylation reaction is catalyzed by the action of transferase enzymes. In this way, **procollagen** is synthesized.

4. Separation of the terminal peptides from procollagen by the action of *protease* enzymes, resulting in insoluble **collagen.**

Following the completion of the four phases of intracellular collagen synthesis, the synthesized collagen, in the form of the fundamental unit—tropocollagen, exits the cell. The stabilization and maturation of collagen fibers occur extracellularly. Stabilization is achieved through the oxidative deamination of NH_2 groups, leading to the formation of aldehyde groups. This reaction is catalyzed by *hydroxylase* enzymes. The resulting aldehyde groups then react with residual NH_2 groups to form Schiff base-type bonds, which possess binding energies comparable to those of peptide bonds.

The maturation of collagen fibers, which proceeds extracellularly, involves the association of the newly formed collagen fibers with glycoproteins such as fibronectin and proteoglycans.

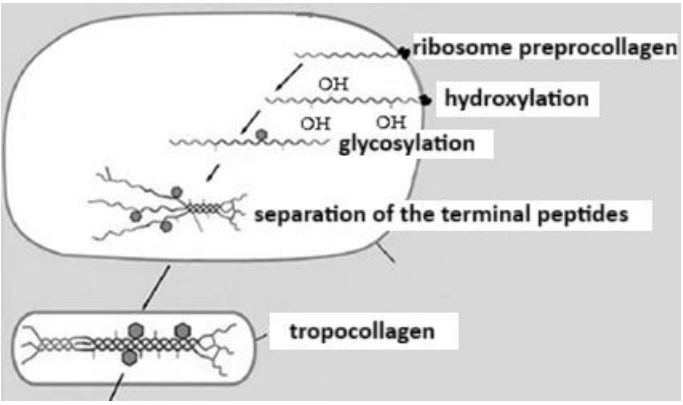


Figure 8.11: *Schematic representation of the intracellular stages of collagen synthesis and its exit from the cell as tropocollagen*

BIOLOGICAL MINERALIZATION

Mineralization refers to the deposition of calcium and phosphate salts during the formation of bone tissue and hard dental tissues.

The concentrations of calcium and phosphate ions in biological fluids vary. Calcium is consistently present as a divalent ion (Ca^{2+}), whereas phosphate ions exist in one of three pH-dependent forms (Figure 8.12): dihydrogen phosphate ($H_2PO_4^-$), monohydrogen phosphate (HPO_4^{2-}), and phosphate (PO_4^{3-}). In solutions with a pH above 6.2, the conversion of calcium dihydrogen phosphate to calcium monohydrogen phosphate predominates. Calcium monohydrogen phosphate is approximately 100 times less soluble than calcium dihydrogen phosphate. The deposited form of calcium phosphate is referred to as apatite.

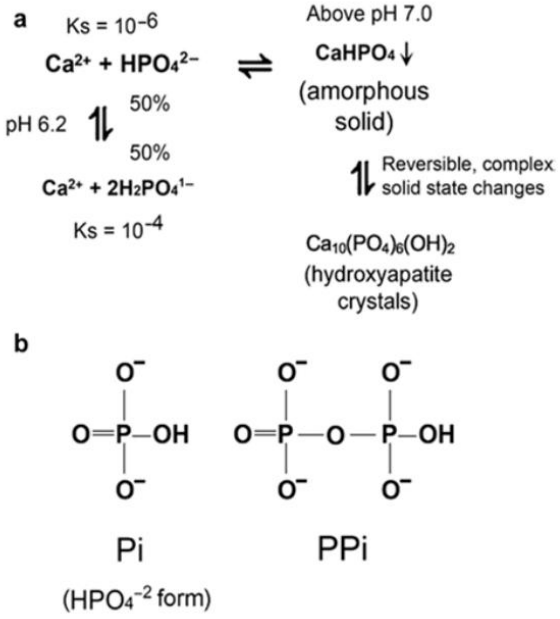


Figure 8.12: *Precipitation of calcium phosphate salts and structure of orthophosphate (Pi) and pyrophosphate (PPi) ions. a) Precipitation of calcium phosphate at different pH values. On the left, at pH 6.2 the fractions of monohydrogen phosphate ions and dihydrogen phosphate ions are almost equal, and the solubility of monohydrogen phosphate is about 100 times lower than the solubility of calcium dihydrogen phosphate. On the right side of the figure it is shown that phosphate ions at pH 7 are more present in the form of monohydrogen phosphate. Calcium monohydrogen phosphate precipitates as an amorphous salt which is further spontaneously remodeled into hydroxyapatite crystals. b) Structure of Pi and PPi. The structures are physiologically ionized forms at pH (~7.2).*

The initially formed apatite is friable and brittle due to its amorphous (non-crystalline) structure. If the surrounding fluid maintains a pH value above 7, this apatite undergoes a series of spontaneous changes that increase its hardness. The final product of calcium and phosphate salt precipitation is hydroxyapatite, a crystalline structure composed of ten calcium ions (Ca^{2+}), six phosphate ions (PO_4^{3-}), and two hydroxyl ions (OH^-).

Hydroxyapatite crystals in bones and teeth also incorporate various other anions and cations, notably magnesium, chloride, carbonate, and fluoride ions. The inclusion of these ions alters the physicochemical properties of hydroxyapatite. The presence of carbonate (CO_3^{2-}) is particularly significant. At low concentrations (<4%), carbonate ions substitute phosphate ions within the crystal lattice, while at higher concentrations (>4%), they replace hydroxyl ions. Both types of substitution result in a slight shortening and thickening of the crystal structure, accompanied by increased solubility. In contrast, replacement of hydroxyl ions by fluoride ions decreases the solubility of apatite.

Crystallographic analysis reveals that in bone and dentin, phosphate is frequently replaced by carbonate, whereas in enamel, it is more commonly substituted by chloride (Cl^-). Carbonated hydroxyapatite plays a crucial role in enamel formation. Enamel contains exceptionally large hydroxyapatite crystals, while bone is characterized by much smaller crystals with numerous vacancies and substitutions. These structural differences enhance the elasticity of bone compared to enamel and facilitate its interaction with surrounding collagen.

Dissolved calcium and phosphate ions in the blood and extracellular fluid remain in solution regardless of their concentration due to the presence of mineralization inhibitors. In blood plasma, mineralization is primarily prevented by polyanions such as albumin, citrate, and pyrophosphate (PPi). These molecules chelate calcium ions, thereby inhibiting their precipitation with monohydrogen phosphate ions.

Pyrophosphate (PPi) inhibits the premature aggregation of calcium ions with monohydrogen phosphate ions in mineralizing tissues and interstitial fluid throughout the body (Figure 8.12, part b). Type I collagen fibers serve as nucleation sites for bone formation under conditions of elevated concentrations of Ca^{2+} and HPO_4^{2-} ions.

Structure of the alveolar bone

Two types of bone tissue are distinguished: dense (compact or cortical bone) and spongy (canalicular or trabecular) bone tissue. The bone matrix is mainly composed of type I collagen fibrils.

Compact bone is composed of numerous osteons, collectively forming the Haversian system (Figure 8.13). Each osteon contains a central canal, known as the osteonic (Haversian) canal, which is encircled by concentric rings (lamellae) of calcified matrix. Bone cells (osteocytes) reside within spaces called lacunae situated between these calcified lamellae. From the lacunae, small canals called canaliculi—containing processes of osteocytes—radiate toward the osteonic canal, facilitating the transport of nutrients and waste. Each osteonic canal houses a central large capillary blood vessel oriented parallel to the long axis of the bone. These capillaries interconnect and, via Volkmann's canals (openings on the bone surface), communicate with larger blood vessels within the thin fibroblast-rich stroma covering the bone surface, known as the periosteum.

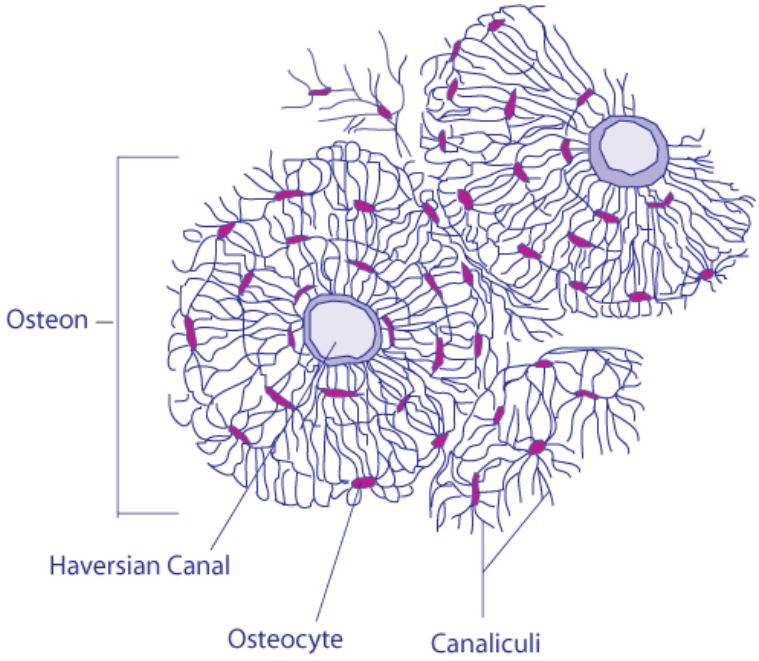


Figure 8.13: *Osteon structure*

Spongy bone is less dense than compact bone and comprises thin trabeculae interspersed with intertrabecular spaces filled with bone marrow (Figure 8.14). The trabeculae are strategically arranged to provide maximal resistance to mechanical forces acting on the bone. They align along lines of pressure. Mechanical stress induces microcracks in the bone, which activate osteoclasts and osteoblasts, leading to targeted remodeling and realignment of the trabeculae. Bone cells differentiate into osteoblasts within the periosteum or on the trabecular surfaces and subsequently become osteocytes residing in lacunae following matrix mineralization. Osteoblasts and osteocytes collectively constitute approximately 15% of the total bone mass.

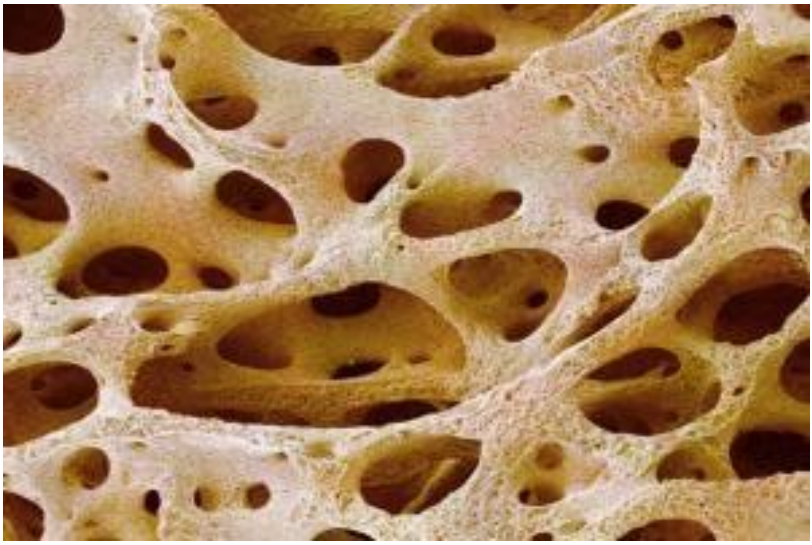


Figure 8.14: *Trabeculae and intertrabecular spaces*

How does biological mineralization take place?

Intramembranous ossification accounts for the majority of mineralization in the skull, including the maxilla and mandible. The process begins with the differentiation and activation of osteoblasts, which originate from fibroblast-like mesenchymal cells within the connective tissue that delineates the future bone formation sites. Osteoblasts secrete a non-mineralized, protein-rich matrix known as osteoid. As osteoblasts migrate, this matrix undergoes mineralization (Figure 8.15). The periosteum, the outermost bone layer, remains uncalcified and harbors latent, undifferentiated osteoblasts responsible for ongoing bone remodeling.

Odontoblasts and cementoblasts also secrete a matrix that resembles osteoid matrix.

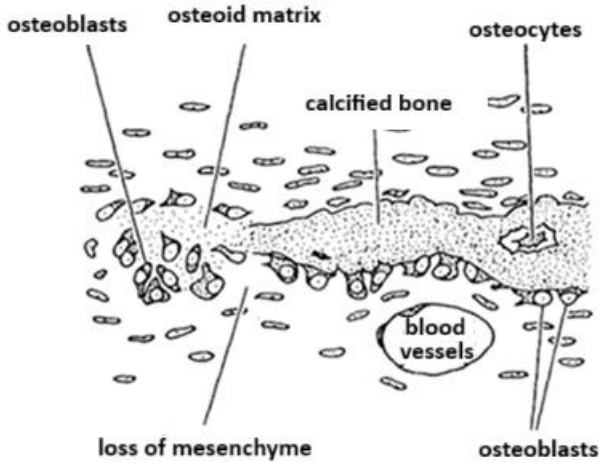


Figure 8.15: *Intramembranous ossification is typical of cranial bones. Osteoblasts differentiate from mesenchymal cells similar to primitive fibroblasts.*

Skeletal tissue mineralization (bone formation) is initiated by osteoblasts that secrete osteoid matrix (Figure 8.16). These cells produce type I procollagen in secretory vesicles.

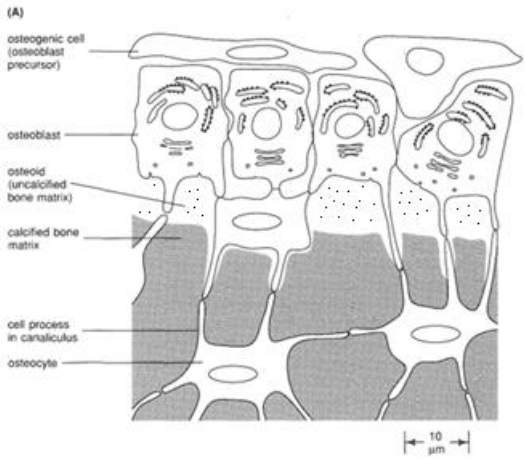


Figure 8.16: *Osteoblast secretion and matrix vesicle formation.*

On the outer surface of the bone are fibroblast-like cells that differentiate into pre-osteoblasts. Osteoblast processes extend into the osteoid tissue and fuse with osteocyte processes (differentiated osteoblasts). The osteoid matrix is filled with small matrix vesicles (black dots in the image) that contain various basic calcium phosphate precipitates.

Osteoblasts transport Ca^{2+} -ions from the periosteal extracellular fluid via Na^{+}/Ca^{2+}- exchangers. The Ca^{2+}- ions are subsequently transferred into the osteoid matrix through the ATP-dependent plasma membrane Ca^{2+}-ATP-ase. In parallel, osteoblasts absorb orthophosphate (Pi) from the periosteal extracellular fluid. At physiological pH (7.0), Pi consists of approximately 60% monohydrogen phosphate and 40% dihydrogen phosphate. Orthophosphate diffuses freely through the cytoplasm and enters the osteoid matrix, specifically the matrix vesicles, where calcification takes place (Figure 8.17). The internal environment of these matrix vesicles is more alkaline due to the activity of carbonic anhydrase, which catalyzes the reaction between dihydrogen phosphate and sodium bicarbonate. The resulting carbonic acid is unstable and rapidly decomposes into water and carbon dioxide, while sodium ions are replaced by incoming Pi in the osteoid matrix. Lactate dehydrogenase is also present and may function to prevent excessive alkalinity, thereby regulating the size of the hydroxyapatite crystals.

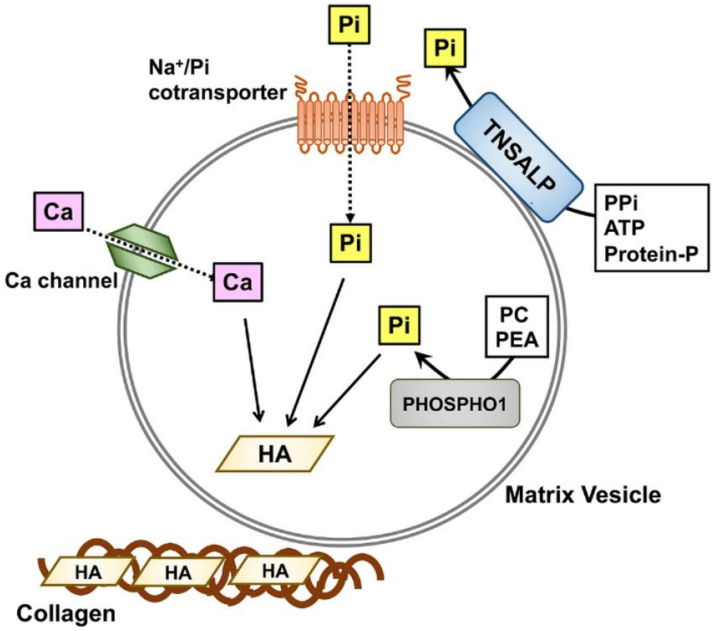


Figure 8.17: *Transport of Ca and orthophosphate into matrix vesicles*

Rapid mineral growth within the matrix vesicle maintains a low concentration of dissolved calcium (Ca^{2+}) and inorganic phosphate (Pi) ions, promoting the continued influx of Ca^{2+} and Pi from the extracellular fluid through their respective transporters. Once the solid calcium phosphate reaches a critical size, the vesicle ruptures, exposing the mineral, which may then partially redissolve.

Collagen is the principal protein constituent of the organic matrix in both bone tissue and dentin. However, several non-collagenous proteins are also present in these mineralized tissues. This group includes **osteocalcin, osteopontin, matrix extracellular phosphoglycoprotein, bone sialoprotein, dentin matrix protein-1, and dentin sialophosphoprotein**. These calcium-binding proteins are primarily localized in the unmineralized or mineralized osteoid and dentin matrix. Non-collagenous proteins play a central role in the ***post-mineralization bone modeling.***

The mineralization of tooth enamel differs fundamentally from that of other oral tissues such as bone, dentin, and cementum. Enamel is formed by ameloblasts, specialized cells that secrete the organic matrix required for enamel mineralization. Unlike the other mineralized tissues, collagen does not take part

in the organic enamel matrix formation. Instead, its principal protein component is *amelogenin*, which is the predominant product of ameloblasts. In addition to amelogenin, the enamel matrix contains smaller amounts of enamelin and ameloblastin. The mineralization of this matrix, occurring exclusively during tooth development, leads to the formation of mature tooth enamel.

Biochemical composition and structure of alveolar bone

Alveolar bone consists of inorganic and organic components, bone cells, and blood vessels. The inorganic portion is primarily composed of calcium phosphate salts, which are present in the form of hydroxyapatite crystals. The predominant organic component is the protein collagen. Other organic constituents of the alveolar bone include chondroitin sulfate and hyaluronic acid.

Bone cells are embedded within the intercellular matrix of the alveolar bone. Among these, osteoblasts are responsible for the formation of alveolar bone and subsequently differentiate into osteocytes. Osteoclasts, in contrast, are involved in the resorption of alveolar bone.

The nutrition of the alveolar bone comes from the blood vessels of the periodontium and from the interdental blood vessels.

The majority of the alveolar bone consists of spongy bone, which is composed of bone trabeculae interspersed with bone marrow. Within the spongy bone are the alveolar sockets (alveoli), which house the roots of the teeth. The walls of the alveoli are formed by compact bone, also referred to as true alveolar bone. On radiographic imaging, this portion of the alveolar bone appears as a thin, radiopaque (white) line surrounding the tooth root and is termed the lamina dura by radiologists. The true alveolar bone contains numerous foramina through which blood vessels and nerve fibers traverse. The ends of the collagenous periodontal fibers are anchored within the alveolar sockets, while their opposite ends are embedded in the cementum of the tooth root. These fibers are essential for securing the tooth within the jaw. The surface of the alveolar bone facing the oral cavity is lined with periosteum.

Alveolar bone remodeling

Mechanism of alveolar bone calcification

Bone calcification begins with the formation of osteoid, a structure that closely resembles cartilage. Osteoblasts synthesize collagen monomers and proteoglycans, which subsequently polymerize to form collagen fibers. Non-collagenous proteins: osteonectin, osteopontin, and osteocalcin facilitate the binding of amorphous calcium salts to the collagen matrix. The initial deposition of calcium results in the formation of amorphous salts, such as primary phosphate and tricalcium phosphate. Continued mineral deposition leads to the development of hydroxyapatite crystals. In addition to synthesizing collagen fibers, osteoblasts also produce substantial quantities of alkaline phosphatase, an enzyme essential for the mineralization of the bone matrix.

The outermost layer of the bone is referred to as the mobile layer, from which calcium bound to amorphous salts (tricalcium phosphate) is most readily released into the extracellular fluid. Osteoblasts and osteoclasts are located on the surface of the bone. These functionally connected cells form a membrane that separates the bone from the extracellular fluid. Between this membrane and the bone lies the bone fluid. Calcium ions are actively transported from the bone fluid into the extracellular fluid via the calcium pump.

This process is known as osteolysis. When the calcium pump is inactivated, the concentration of calcium in the bone fluid rises, leading to the re-deposition of calcium into the bone matrix. This exchange of calcium in the cellular layer of bone has a buffering role because it does not allow the concentration of calcium in the extracellular fluid to decrease or increase.

Mechanism of bone breakdown (resorption)

The cells that participate in the breakdown of bone are called osteoclasts. They have extensions and secrete into the bone:

- proteolytic enzymes (originating from lysosomes) that break down the organic matrix and
- citric and lactic acids that break down bone salts.

Bone remodeling processes

Bone formation occurs over a period of 8 to 10 weeks, while bone resorption takes place more rapidly, within 3 to 4 weeks. Both processes allow the bone's strength to adapt to the degree of stress placed. However, the rate of these processes decreases with age. In young individuals, 100% of calcium is replaced within a year, whereas in adults, only about 18% of calcium undergoes replacement annually. These processes are influenced by the direction and intensity of the forces acting on the teeth. Bones thicken in response to increased loading; however, if the applied load exceeds the endurance capacity of the alveolar bone, bone resorption occurs. Bone remodeling leads to changes in bone shape, allowing it to better withstand mechanical forces. In dentistry, these remodeling processes are utilized for orthodontic tooth movement, while in oral and maxillofacial surgery, distractors are used to shape the jawbones accordingly.

9 BIOCHEMICAL CHARACTERISTICS OF DENTAL TISSUES

TOOTH ENAMEL (SUBSTANTIA ADAMANTINA)

- **Changes in the composition of hydroxyapatite**
- **Role of salivary electrolytes in the preservation of tooth enamel**

DENTIN

CEMENTUM

DENTAL PULP

There are three hard dental tissues: tooth enamel, dentin, and cementum. The soft dental tissue that lies within these hard dental tissues is called the dental pulp.

The tooth enamel (enamelum) is the outermost layer of the tooth and covers the crown. Dentin (dentinum) is the most abundant dental tissue and is located between the enamel and the dental pulp. Cementum is the third dental tissue, covering the dentin in the root portion of the tooth. The dental pulp is the vital part of the tooth and is located within the centrally positioned tooth cavity (cavum pulpae) (Figure 9.1).

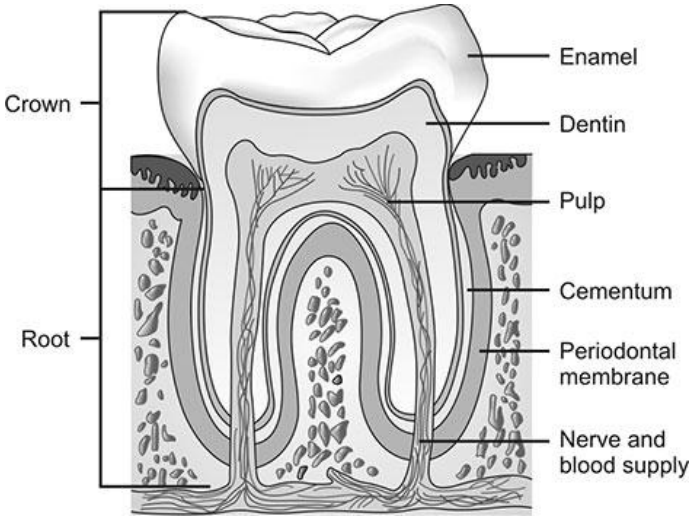


Figure 9.1: *Longitudinal section of the tooth*

TOOTH ENAMEL (SUBSTANTIA ADAMANTINA)

Tooth enamel is the most superficial hard dental tissue and covers the dentin in the coronal part of the tooth. It is produced by ameloblast cells in a process called amelogenesis. After amelogenesis is complete, the ameloblasts disappear. Enamel is the hardest tissue in the human body, a characteristic that results from its highly specific biochemical composition.

Enamel is a tissue with the highest proportion of inorganic substances in the body, comprising about 95% of its composition. These inorganic substances are mainly calcium and phosphate ions arranged in the crystal lattice structure of hydroxyapatite. Water content in tooth enamel is very low, approximately 4%, making enamel the tissue with the lowest water content in the human body. Of this, 0.8% is labile bound water associated with the organic components of enamel, while 3.2% is hydration water bound to the hydroxyapatite crystals. Organic substances constitute a small fraction, about 1.1–1.3%, of the enamel's composition.

Enamel possesses a crystal lattice structure composed of hydroxyapatite crystals, which closely resemble natural apatite. During amelogenesis rapid crystallization may lead to the formation of small hydroxyapatite crystals, whereas slower crystallization results in larger macrocrystals that exhibit greater resistance to dental caries.

The empirical formula of hydroxyapatite (HA), the primary component of enamel, is $Ca_{10}(PO_4)_6(OH)_2$. Hydroxyapatite crystals have the shape of hexagonal prisms, where three calcium ions form triangular arrangements. Within each triangle, the phosphate group is located centrally. The oxygen atom of the hydroxide ion (OH^-) sits above the plane of this triangle, with the hydrogen atom positioned below the oxygen. Hydrogen bonds form between the oxygen of one triangle and the hydrogen of an adjacent triangle located above it. Two such triangles combine to create a hexagonal prism, exhibiting a hexagonal arrangement of Ca^{2+} ions within the crystal lattice. Within these hexagonal formations, calcium ions (Ca^{2+}) occupy two distinct positions: columnar Ca^{2+} ions are situated at the vertices of the hexagon, while axial Ca^{2+} ions are located inside the crystal structure of the hydroxyapatite, arranged in a triangular pattern.

Phosphorus is present in the form of phosphate ions (PO_4^{3-}). The four oxygen atoms of the phosphate group are arranged in a square shape, with the phosphorus atom located at the center of this square. Between every two columnar Ca^{2+} ions, there are two phosphate groups. The orientation of these phosphate groups is distinctive: every second phosphate group protrudes above the plane, connecting two calcium ions through three oxygen atoms and one phosphorus atom. This arrangement results in the surface of the hydroxyapatite crystals, and consequently the entire tooth enamel, carrying a negative charge (Figure 9.2).

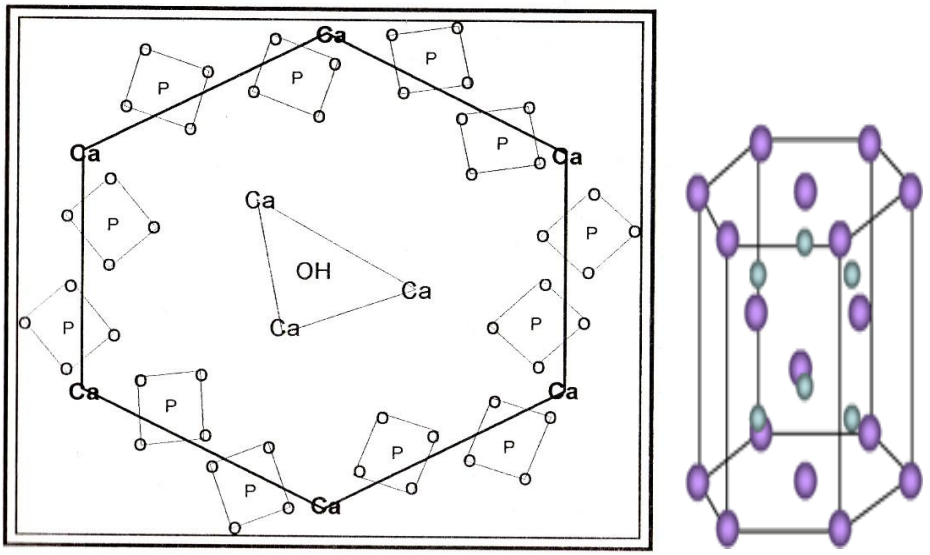


***Figure 9.2: Left**: Cross-section of hydroxyapatite and the spatial arrangement of its ions. **Right**: Hexagonal shape of hydroxyapatite*

Biochemical analysis of hard dental tissues has confirmed the presence of various microelements, with tooth enamel being the most commonly examined tissue. More than 40 microelements have been identified in dental tissues. Their concentration largely depends on the amount of these microelements ingested by the body. Besides intake, the concentration also varies according to the type of hard dental tissue, as well as the age and gender of the individual. Generally, microelement concentrations are higher in tooth enamel compared to dentin, although this is not always the case. Notably, enamel contains higher levels of manganese (Mn), copper (Cu), strontium (Sr), lead (Pb), and zinc (Zn) compared to dentin. In dentin, the most abundant microelements are magnesium (Mg),

fluoride (F), and iron (Fe). Differences in microelement concentrations are also observed between individuals of different ages. For example, the enamel of older individuals contains significantly higher levels of copper (Cu), strontium (Sr), and lead (Pb) compared to younger individuals. Similarly, dentin in older individuals shows notably higher concentrations of zinc (Zn) and lead (Pb). Additionally, the enamel of teeth in females exhibits significantly higher concentrations of zinc (Zn) and selenium (Se) compared to males (Table 9.1).

Table 9.1: Division of microelements according to their content in the surface layer of tooth enamel

Group	Concentration µg/g	Microelements
I	<0,1	Bi, I, Mo
II	0,1-1,0	Co, Pr, Cs, Rb, Zr
III	1,1-10,0	Ce, La, Cr, V, Ti, Be, Sn, Cd, Br, Ge
IV	10,1-100,0	Ba, Ag, Ni, Li, Ga, Pb, Sb, Se, Mn
V	100,1-1000,0	Fe, Sr, Cu, Al, Mg, Zn
VI	>1000	F, S

Hydroxyapatite crystals are the most abundant component in tooth enamel, as well as in other hard dental tissues such as alveolar bone. In tooth enamel, these crystals are specifically organized into a distinct anatomical structure known as the enamel prism. Enamel prisms are the fundamental anatomical units of tooth enamel and are unique to this tissue, as they are absent in dentin and cementum. The enamel prisms measure approximately 5 µm in diameter and are arranged in rows extending from the enamel-dentine junction to the enamel surface. Prisms are absent in the most superficial layers of enamel as well as at the enamel-dentine junction itself; these regions are known as aprismatic layers. The aprismatic layers have a thickness of 20–30 µm, with

hydroxyapatite crystals oriented perpendicular to the enamel surface. The shape of enamel prisms resembles a “key” or a “fish.” Each enamel prism consists of two anatomical parts: the head and the tail. The heads are oriented toward the masticatory (biting) surface of the tooth, while the tails face toward the root. The head of one prism interlocks with the tails of two adjacent prisms, and conversely, the tail of one prism lies between the heads of two neighboring prisms. Despite this close articulation, microspaces called interprismatic spaces exist between the prisms (Figure 9.3).

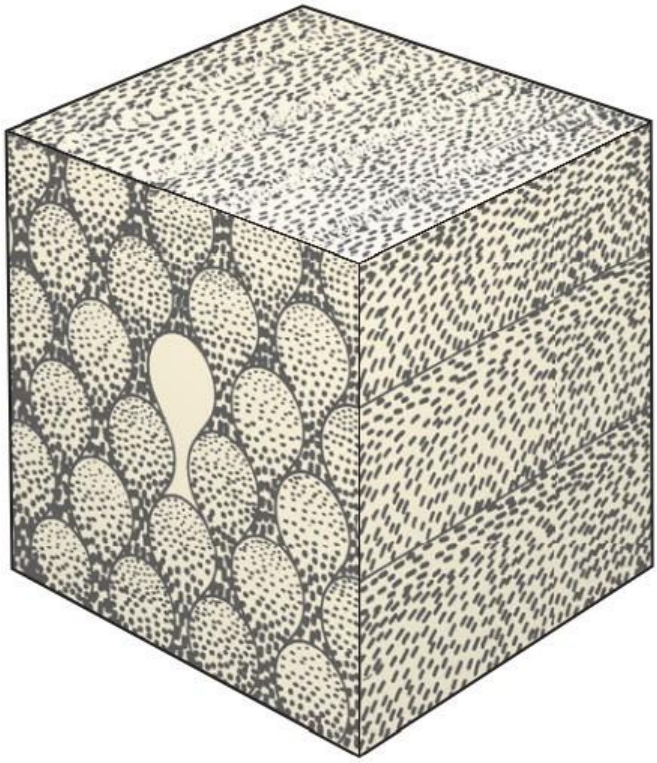


Figure 9.3: *Schematic representation of enamel prisms*

In addition to inorganic substances, organic substances are also present in tooth enamel. Notably, tooth enamel is the only hard tissue in the human body that lacks collagen in its organic matrix, unlike bone, dentin, and cementum where collagen predominates. Specific proteins unique to enamel have been identified among its organic components. These proteins are synthesized during amelogenesis by ameloblasts and include hydrophobic proteins called amelogenins, as well as non-amelogenin proteins, also known as anionic enamel proteins, such as ameloblastin (or amelin), enamelin, and enamelisins. These enamel-specific proteins play crucial roles in the mineralization process of tooth enamel.

The most abundant proteins in tooth enamel are *amelogenins*, comprising approximately 90% of the total protein content in this dental tissue. These proteins belong to the group of hydrophobic proteins and are rich in the amino acids proline, glutamine, leucine, and histidine. *Ameloblastin*, also known as *amelin*, is an anionic protein characterized by a high content of proline, glycine, and leucine. *Enamelin* is the largest protein in tooth enamel, with a molecular mass of about 32 kDa. It contains both hydrophobic acidic and basic domains located in different regions of the molecule.

Changes in the composition of hydroxyapatite

Changes in the composition of hydroxyapatite occur for two main reasons: adsorption and ionic exchange.

Adsorption primarily affects the most superficial layers of enamel, as these layers are in direct contact with saliva components and are therefore more susceptible to such changes. The process of adsorption is facilitated by the negative charge present on the surface of hydroxyapatite crystals.

It is clear that adsorption of positively charged ions (cations) occurs easily because they bind to the residual negative charge of the phosphate groups in hydroxyapatite. However, anions can also be attracted and held by these adsorbed cations, especially divalent and trivalent ones. As a result, an adsorbed ionic layer forms on the surface of hydroxyapatite crystals. This layer can be removed if the local pH changes, causing the desorption of all charged particles.

When an adsorbed ionic layer forms on the surface of hydroxyapatite crystals, it facilitates the binding of a certain amount of water, creating a hydration layer. This hydration layer enables changes in the overall composition of the hydroxyapatite.

Ionic change represents another form of alteration in the composition of hydroxyapatite, occurring through the exchange of ions between the hydroxyapatite crystal lattice and its surrounding environment. It is estimated that, under certain conditions, up to one third of the ions within the hydroxyapatite lattice can be replaced. Two distinct types of ionic change are recognized: isoionic and heteroionic.

Isoionic change does not alter the natural properties of the hydroxyapatite crystal because, during this process, calcium ions within the hydroxyapatite lattice are replaced by calcium ions from the surrounding environment, such as saliva. Although this process occurs very slowly, it is continuous in the oral environment and has been demonstrated experimentally using labeled calcium ions.

Heteroionic change takes place when calcium ions in the hydroxyapatite crystal lattice are replaced by other divalent cations such as manganese, magnesium, or sometimes by sodium or hydronium ions (H_3O^+). This substitution alters the overall composition of the crystal, leading to the loss of its natural properties, particularly increasing its solubility. Additionally, during heteroionic change, the total water content in enamel can rise because one calcium ion may be replaced by two hydronium ions. These changes typically occur when the mineralization process of enamel and other hard dental tissues is disrupted, often due to vitamin deficiencies or hormonal imbalances.

Heteroionic changes in the composition of hydroxyapatite are generally harmful and undesirable, except when the hydroxyl group is replaced by fluorine. This substitution forms fluorapatite (Figure 9.4), which significantly stabilizes the crystal structure. The hydroxyl group is a dipolar ion and binds surrounding calcium ions with varying strengths, contributing to the instability of hydroxyapatite and allowing for various heteroionic or isoionic substitutions. In contrast, fluorine is a much more compact anion that binds the surrounding calcium ions uniformly, thereby preventing their replacement and enhancing the stability of the hydroxyapatite crystal.

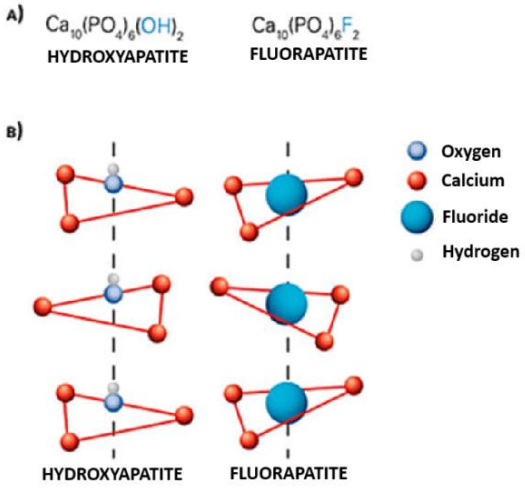


Figure 9.4: *Positive heteroionic shift*

Role of salivary electrolytes in preserving tooth enamel

Saliva contains numerous electrolytes, including bicarbonates, calcium, chlorides, fluorides, iodides, magnesium, phosphates, sodium, potassium, sulfates, and thiocyanates. These electrolytes enter the saliva through ultrafiltration from the blood plasma. Notable differences exist between the concentrations of these electrolytes in blood plasma and in saliva. Additionally, the electrolyte concentrations vary between unstimulated saliva and stimulated saliva (Table 9.2).

Table 9.2: Concentration of individual electrolytes in stimulated saliva, in unstimulated saliva and in blood plasma

Electrolytes	Unstimulated saliva mmol/L	Stimulated saliva mmol/L	Blood plasma mmol/L
Na^+	15	30	140
Cl^-	23	40	105
Ca^{2+}	2	4	2,5
K^+	25	4,5	4,5
HCO_3^-	1	60	27
$H_2PO_4^-$	7-8	2-3	4

Salivary electrolytes play a unique and indispensable role in the complex mechanism of enamel preservation. Their specific composition and balance are essential for protecting hard dental tissues, particularly tooth enamel, from dissolution and demineralization.

Water is a fundamental component of all body fluids, including saliva, where it supports numerous functions within the oral cavity. Due to its nature as a universal solvent, water can dissolve hard dental tissues, particularly tooth enamel, which is constantly exposed to saliva and its irrigating and dissolving effects. To counteract the latter, saliva contains electrolytes. These electrolytes, present in specific concentrations and ratios, help establish a balance with the minerals deposited in tooth enamel, thereby protecting it from excessive dissolution.

Recent research shows that saliva is oversaturated with specific ions—particularly calcium, phosphate, and hydroxyl ions—which are fundamental components of the hydroxyapatite crystal lattice in tooth enamel. This indicates the existence of a finely controlled balance between the electrolyte content in the salivary fluid and that deposited within the enamel. The direction of this balance depends on the pH of the saliva: it can shift either toward dissolution and demineralization or toward deposition and remineralization of the tooth enamel.

In conditions of increased salivation, there is a substantial rise in the total volume of saliva produced, accompanied by an increased concentration of salivary electrolytes such as calcium, phosphate, and hydroxyl ions. Additionally, bicarbonate levels rise significantly, leading to an inevitable increase in saliva pH. Under these circumstances, the electrolyte balance shifts toward deposition, promoting the remineralization of tooth enamel.

After the cessation of masticatory function, the volume of saliva production decreases, leading to a reduction in the total amount of saliva and the concentration of excreted electrolytes. Bicarbonate levels drop to minimal values, while phosphate concentration increases significantly. Consequently, the pH of saliva decreases, acidity rises, and conditions favor a shift in the electrolyte balance toward dissolution, i.e., demineralization. However, this process is not widespread but localized to specific microregions where bacterial colonies (dental plaque) form. In these microareas, due to poor oral hygiene and excessive sugar intake, the concentration of acidic metabolites—particularly lactate—increases, causing the local pH to fall below 5.5. This acidification promotes an unfavorable shift in the electrolyte balance, leading to the dissolution and demineralization of tooth enamel.

Numerous studies have demonstrated that chewing gum, whether containing sugar or not, positively influences the balance of electrolytes in enamel and saliva, thereby benefiting dental health. Chewing stimulates the salivary reflex, which increases saliva production. This increase leads to a higher concentration and total amount of electrolytes, an elevated bicarbonate concentration, and a rise in pH. These changes positively affect the mineral content of tooth enamel. Additionally, the harmful effects of sugar consumption and its breakdown into lactate are mitigated because lactate is neutralized by the bicarbonates secreted in saliva. Consequently, the equilibrium between salivary electrolytes and tooth enamel shifts favorably toward remineralization.

It is known that salivary secretion contains several protein fractions that show affinity for calcium. Such proteins include statherin, various

phosphoproteins, and proline-rich proteins. It is assumed that with the help of these protein carriers, in the area of the enamel lesion that needs to be remineralized and recrystallized, the critical concentration of calcium and phosphates is ensured. Calcium and phosphates, at a certain pH value of the local environment (alkaline), are released and mutually react, forming appropriate compounds.

The need for remineralization arises in microregions of the tooth enamel where damage has occurred, meaning that part of the mineral content has been lost. In these areas, the enamel is "bare." Because the hydroxyapatite crystals in enamel carry a negative charge, these exposed regions become covered by a dental pellicle, especially during mastication. The dental pellicle primarily consists of salivary glycoproteins, within which numerous calcium ions are embedded. Additionally, the microspaces between the adsorbed salivary glycoproteins contain salivary fluid that has the typical ionic composition of saliva found in the oral cavity.

The oral environment contains numerous and diverse mechanisms that are reflexively stimulated or inhibited in order to preserve both dental health and overall oral health.

DENTIN

Dentin is the second hard dental tissue and the most abundant among all hard dental tissues. The cells responsible for producing dentin are called odontoblasts, which originate from the tooth pulp. The process of dentinogenesis, or dentin formation, continues throughout an individual’s life as long as the tooth remains vital.

Dentin differs markedly from tooth enamel in its biochemical composition. It contains a significantly higher proportion of water, approximately 10%, and a greater percentage of organic substances, about 20%. The remaining 70% consists of inorganic substances, primarily calcium ions (Ca^{2+}) and phosphates, which are present as hydroxyapatite crystals. Among the organic components of dentin, collagen is the most abundant structural protein, making up around 18%. Collagen is also the main organic substance in the third hard dental tissue, cementum.

In the organic matrix of dentin, besides collagen, there are also “non-collagenous proteins” such as dentin phosphoprotein, dentin sialoprotein, and dentin matrix protein. These phosphorylated proteins play a crucial role in the

mineralization process during dentinogenesis. Like collagen, they are synthesized by odontoblasts.

Besides its biochemical characteristics, dentin also differs from enamel in its histological structure. Histological sections of dentin reveal dentinal tubules that run transversely through the full thickness of the dentin, extending from the enamel-dentin junction to the dental pulp. These tubules have a conical shape, being narrowest near the enamel-dentin junction and widest near the dental pulp (Figure 9.5). The area between the tubules is called intertubular dentin, which constitutes the majority of dentin. Inside the tubules lies the intratubular dentin. This type of dentin, known as intratubular dentin, is better mineralized compared to the dentin located between the dentinal tubules (intertubular dentin). Because of its specific tubular structure, dentin has weaker barrier properties than tooth enamel. Enamel is impermeable and serves as an excellent protective barrier. The barrier function refers to preventing the penetration of antigens from the external environment into the inner dental tissues. The excellent barrier properties of enamel are maintained only when it is healthy and intact. However, when enamel is damaged, most commonly due to tooth decay, the underlying dentin becomes exposed to the external environment. In such cases, the dentinal tubules provide a relatively easy pathway for antigens to reach the vital dental tissue, the dental pulp, increasing the risk of irritation or infection.

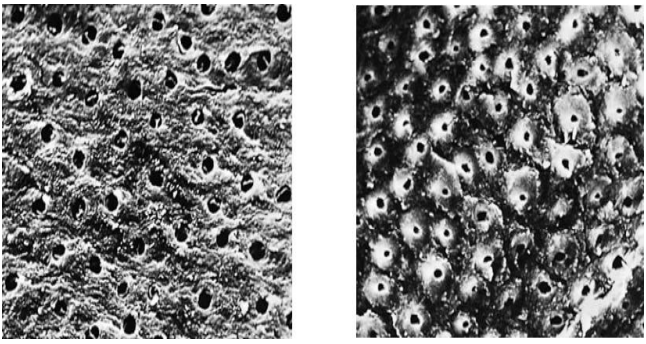


Figure 9.5: *Left: Dentinal tubules near the dental pulp*

Right: Dentinal tubules near the enamel-dentin junction

Another significant difference between tooth enamel and dentin lies in their formation timelines. Tooth enamel is formed only once in a lifetime during tooth development within the alveolar bone, through a process called amelogenesis. After the tooth erupts, enamel formation ceases permanently. In contrast, dentin is synthesized not only during tooth development

(dentinogenesis) but can also continue to be produced later in life. The dentin formed during dentinogenesis is called primary dentin and is histologically characterized by its specific tubular structure with numerous dentinal tubules. Throughout life, two additional types of dentin can be synthesized: secondary dentin and tertiary dentin. Both secondary and tertiary dentin are synthesized by specialized dental pulp cells called odontoblasts, in response to specific physiological or non-physiological stimuli affecting the pulp tissue. Secondary dentin is produced by odontoblasts in response to the normal physiological stimuli of the pulp. These stimuli include mechanical factors, such as pressure on the biting surface during mastication, and thermal factors, such as exposure to hot or cold foods. Consequently, the dentin formed under these conditions is termed physiological or secondary dentin. According to its histological characteristics, secondary dentin differs from primary dentin by having a significantly smaller number of dentinal tubules. Additionally, these tubules are narrower in diameter and more curved. As a result, secondary dentin considerably reduces the potential for antigens from the external environment to penetrate into the dental pulp. Tertiary dentin, on the other hand, is produced by odontoblasts in response to non-physiological stimuli, typically associated with damage or loss of tooth enamel. This often occurs during the carious process, when dentinal tubules become exposed to external influences. Various stronger stimuli are transmitted to the dental pulp through the open dentinal tubules. In response, the pulp "defends" itself by synthesizing new layers of tertiary dentin. Tertiary dentin is characterized by having the lowest density of dentinal tubules. Its tubules are significantly fewer in number, and there are areas completely devoid of tubules. Consequently, tertiary dentin provides effective barrier properties that help prevent the penetration of antigens. However, the pulp's defensive capacity is limited. Often, antigens, primarily microorganisms, manage to penetrate into the dental pulp, leading to an inflammatory process known as pulpitis.

CEMENTUM

Cementum is the third hard dental tissue and covers the dentin in the root portion of the tooth. It consists of approximately 12% water, 23% organic substances, and 65% inorganic substances. The inorganic component is primarily composed of hydroxyapatite crystals, while collagen is the dominant substance in the organic portion.

There are two types of cementum: acellular (primary) and cellular (secondary). Acellular cementum lacks cells, specifically cementoblasts, and extends along the entire root surface. It is formed during the process of cementogenesis. Cellular cementum, on the other hand, contains cementoblasts that participated in its formation. This type is found near the apex of the root and overlays the acellular cementum (Figure 9.6). Cementoblasts are responsible for synthesizing secondary cementum. This type of cementum is continuously formed throughout life, regardless of whether the tooth is vital (the pulp is preserved) or non-vital (the pulp has been removed due to an inflammatory process). This independence from the dental pulp is because cementoblasts obtain nutrients from the periodontal fluid, which fills the periodontal space (spatium periodontale) located between the tooth root and the alveolar bone.

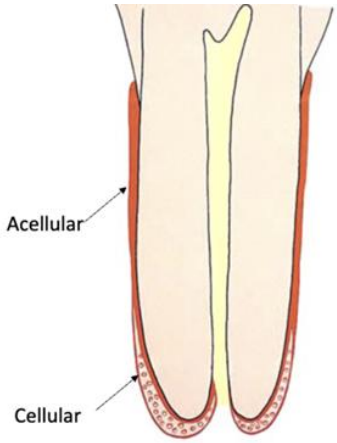


Figure 9.6: *Acellular (primary) and cellular (secondary) cement*

The inner surface of the cementum is firmly attached to the dentin. Its outer surface is where the periodontal fibers attach, thus securing the tooth in the jaw. The other end of the periodontal fibers is connected to the alveolar bone.

Biochemically, cementum is similar to bone tissue. However, a key difference exists between the two: bone is vascularized (contains blood vessels) and innervated (contains nerve fibers), whereas cementum is avascular and lacks innervation. Due to this, cementum is resistant to mechanical pressure and forces. When mechanical forces act on bone, resorption occurs on the side where the force is applied, while bone formation (apposition) takes place on the opposite side. These remodeling processes are not typical for cementum. Orthodontic therapy for the correction of malpositioned teeth in the jaw is based on this property of cementum. Specifically, the application of appropriate orthodontic appliances, which allow the exertion of mechanical forces, stimulates resorption of the alveolar bone. The cementum, in this process, undergoes no changes, which enables the teeth to move and be brought into the proper position.

Secondary cementum, as stressed previously, is continuously synthesized throughout life, specifically at the very tip of the tooth root. By forming new layers of secondary cementum at the root apex, the tooth gradually moves toward the oral cavity, upper teeth move downward, and lower teeth move upward. This movement compensates for the loss of tooth enamel caused by attrition. Although the enamel of the tooth is protected from excessive wear during mastication by the dental pellicle, this harmful process still occurs to some extent. Thus, with aging, the superficial layers of the tooth enamel are “worn down” as a result of the existing attrition. Without the ongoing synthesis of secondary cementum, the contact between opposing teeth in the upper and lower jaws would be lost, disrupting normal chewing function. However, this does not occur because secondary cementum is synthesized to the extent that the tooth enamel is worn down as a result of attrition. This phenomenon is also known as the “permanent tooth eruption.” Hence, the contribution of cementum to the maintenance of oral homeostasis arises, enabling the preservation of contact between antagonist teeth (the teeth of the upper jaw and the corresponding teeth of the lower jaw).

DENTAL PULP

The dental pulp is the soft, vital tissue located in the central cavity of the tooth, known as the cavum pulpae. It consists of loose connective tissue containing the pulp’s basic substance, various cell types, blood vessels, nerve fibers, and collagen fibers. The pulp performs three primary functions: nutritional, by nourishing dental tissues through its blood vessels; sensory, by providing tooth sensitivity via its nerve fibers; and defensive, through specialized cells that protect against harmful agents.

ORAL BIOCHEMISTRY AND PHYSIOLOGY

The biochemical characteristics of the pulp show that it is made up of 75% water and 25% organic matter.

The ground substance of the pulp has a gelatinous appearance and high diffusion capacity. Due to these high diffusion properties, rapid exchange of metabolites and nutrients occurs throughout the pulp. The ground substance of the pulp is composed of various proteins and glycoproteins, particularly glycosaminoglycans such as hyaluronic acid and chondroitin sulfate in a proteoglycan combination..

Proteins present in the pulp are: collagen, fibronectin, chondroitin sulfate, dermatin sulfate, keratin sulfate.

Odontoblasts, fibroblasts, and defense cells are present in the dental pulp. Odontoblasts are specialized pulp cells located adjacent to the dentin wall of the cavum pulpae. These cells participate in the formation of secondary and tertiary dentin throughout life as a response of the pulp to various physiological and non-physiological stimuli. In this way, the pulp “defends” itself against antigen penetration and potential inflammation. Due to the synthesis of new layers of secondary and tertiary dentin, the volume of the pulp gradually decreases with age; consequently, the pulp in children and adolescents is large and voluminous, whereas in older individuals it is smaller and may even be reduced to a narrow slit. Beneath the odontoblast layer, there is an acellular zone known as the Weil zone. Below this zone are fibroblasts, which are the most numerous cells in the pulp and synthesize collagen fibers. This zone also contains defense cells, blood vessels, and nerve fibers. Defense cells protect the pulp from antigens. Representatives of defense cells in the pulp include leukocytes, mast cells, histiocytes, and others.

10 STRUCTURE AND FUNCTION OF THE ACQUIRED DENTAL PELLICLE

DENTAL PELLICLE COMPOSITION

Proteins

Carbohydrates

Lipids

Functions of the dental pellicle

Lubrication of the tooth surface

The dental pellicle as a semipermeable membrane

Mineral homeostasis

Modulation of bacterial adherence

The formation of the acquired dental (salivary) pellicle results from the selective adsorption of salivary biopolymers onto the surface of the tooth. This pellicle is composed of specific proteins and other macromolecules that originate from the local oral environment, particularly from saliva and gingival crevicular fluid and is different from the bacterial biofilm (plaque). The formation process is highly selective—only certain fractions of the proteins found in saliva are incorporated into the pellicle structure.

The first phase of pellicle formation, known as **the initial phase**, is marked by the rapid adsorption of salivary proteins onto the enamel surface of the tooth. This process occurs within just a few minutes after the tooth is exposed to saliva. During this short time, a thin protein layer forms on the enamel, typically measuring between 10 and 20 nanometers in thickness.

The initial stage of dental pellicle formation involves the selective adsorption of salivary proteins onto the enamel surface. Upon contact with salivary electrolytes, calcium ions from the enamel crystal lattice demonstrate a higher solubility than phosphate ions. Consequently, phosphate ions remaining on the enamel surface impart an overall negative charge. This negatively charged surface becomes coated with positively charged calcium ions originating from saliva. The primary adsorption of salivary proteins occurs through electrostatic (ionic) interactions between the ionic bilayer (composed of calcium and phosphate ions) and the oppositely charged groups of salivary proteins (Figure 10.1).

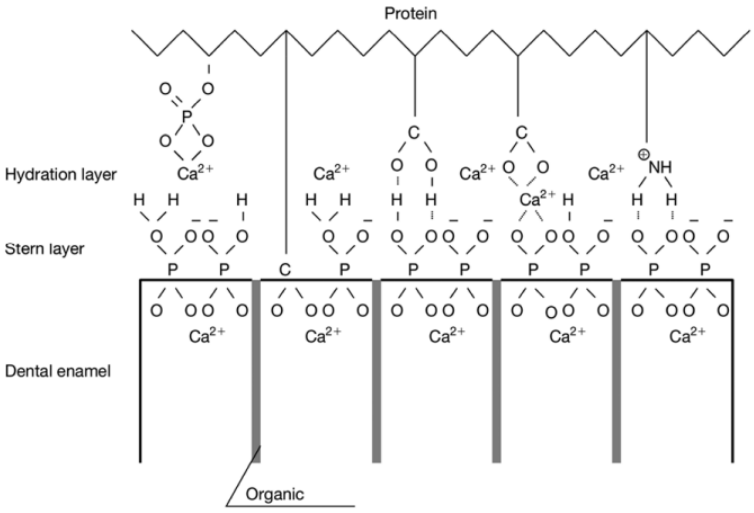


Figure 10.1: *Schematic representation of pellicle formation by adsorption of salivary proteins onto the tooth enamel surface through various electrostatic interactions*

Salivary proteins with a high affinity for hydroxyapatite in tooth enamel are referred to as "pellicular precursor proteins." Phosphoproteins such as statherins, histatins, and proline-rich proteins exhibit strong binding affinity to hydroxyapatite and are the first salivary proteins to adsorb to its surface. The initial layer of the dental pellicle, formed within 30 seconds to 3 minutes, comprises a more diverse and heterogeneous array of biomolecules than previously assumed. Electrophoresis has identified more than 10 distinct proteins in the initial dental pellicle, including mucin type 1 and type 2, amylase, statherin, histatin, multiple forms of cystatin, lysozyme, lactoferrin, carbonic anhydrase 1 and 2, and glucosyl transferase.

Given the involvement of a wide range of salivary proteins in the initial pellicle formation, it is understandable that their attachment to the tooth enamel surface is not solely mediated by ionic interactions. Other ways of binding salivary proteins to the tooth enamel surface include Van der Waals interactions and hydrophobic interactions. These latter interactions are thermodynamically driven, resulting from the energy gained during the adsorption of protein pellicular precursors. The rapid initial phase of salivary protein adsorption is followed by the secondary phase of dental pellicle formation.

The secondary phase of dental pellicle formation is characterized by the slower adsorption of salivary proteins onto the already established initial protein layer on the tooth enamel surface. This phase involves a continuous process of

biopolymer adsorption from saliva. This process is characterized by protein-protein interactions between proteins already adsorbed in the initial pellicle layer and protein aggregates from saliva. Analysis of the amino acid composition of the dental pellicle has confirmed that the pellicle thickness, established early (within 2–3 minutes), remains stable for the subsequent thirty minutes. The thickness of the pellicle subsequently increases approximately threefold, reaching its maximum around the 90th minute after formation begins. Within 60 minutes, the pellicle thickness increases from 100 to 1000 nm, depending on the concentration of biopolymers in saliva and the conditions within the oral cavity.

The increase in pellicle thickness between 30 and 90 minutes is attributed to the adsorption of protein aggregates from saliva rather than individual salivary proteins. It has been demonstrated that most proteins in parotid saliva exist as aggregates with a globular structure, measuring 100 to 200 nm in diameter. These globules possess a hydrophobic interior and a negatively charged surface, resembling milk micelles, and are referred to as micelle-like structures. The presence of various complexes of salivary mucins and other salivary proteins within the pellicle has also been demonstrated. Some of these complexes form prior to, while others form after, their adsorption onto the hydroxyapatite of tooth enamel. These complexes (aggregates) of salivary proteins consist of non-covalently linked amylase, proline-rich proteins (PRP), histatins, statherin, cystatins, and lysozyme on one hand, and MG1 (mucin type 1) on the other. This evidence indicates that the formation of the dental pellicle is primarily driven by the adsorption of protein aggregates rather than individual salivary proteins. The enzyme transglutaminase, produced by buccal epithelial cells, plays a key role in the formation and cross-linking of salivary protein aggregates. These aggregates serve as a reservoir of protein precursors for the dental pellicle and offer protection against proteolytic degradation (Figure 10.2).

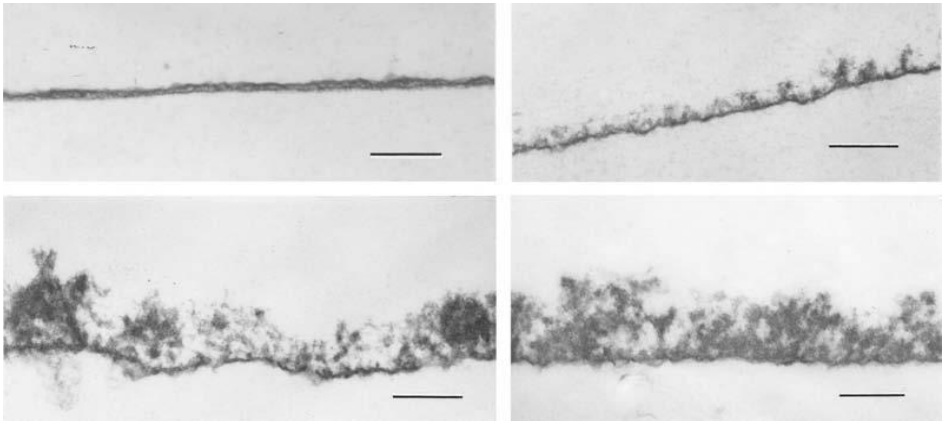


Figure 10.2: *Photomicrograph of pellicle layers 1 minute (top left), 10 minutes (top right), 60 minutes (bottom left) 120 minutes (bottom right) created on tooth enamel on the buccal surface of an upper first molar.*

The formation of the pellicle is a dynamic process involving both adsorption and desorption, influenced by bacterial and host enzymes. The limiting factors and parameters that determine the balance between these processes remain unclear.

COMPOSITION OF THE DENTAL PELLICLE

The results of studies on the composition of the dental pellicle are contradictory. It is assumed that the pellicle layer, which forms on the labial surface of the teeth, contains about 1 ng of proteins over a period of 2 hours.

In the 1970s, it was suggested that there were no differences in the composition of the dental pellicle, regardless of which tooth surface or region of the mouth it was formed on. The amino acid composition of the pellicle, examined from the buccal surfaces of the upper molar and upper incisor and the lower front teeth, was identical. Research conducted during this period also indicates that the amino acid composition of the pellicle does not differ between different individuals.

In later studies on the composition of the pellicle, conducted using the protein electrophoresis method, it was confirmed that the pellicle's composition is determined by the characteristics of the saliva with which the tooth comes into contact. These findings indicate that the local availability of specific salivary biopolymers significantly influences the composition of the dental pellicle.

In recent years, methods for collecting the pellicle have been refined by combining mechanical and chemical removal. The pellicle is collected from the tooth surface using a filter membrane (polyvinylidene fluoride) immersed in a 0.5 molar solution of sodium bicarbonate.

Proteins

The protein composition of the dental pellicle is examined through amino acid analysis using immunological, histochemical, chromatographic, and electrophoretic methods. It has been confirmed that the proteins forming the dental pellicle are, in fact, proteins and glycoproteins present in salivary secretion. These are given in the following table (Table 10.1).

Table 10.1: Proteins and other biopolymers present in the dental pellicle, formed in vivo on the surface of tooth enamel

Biopolymers	Physiological role	Molecular mass (kDa)
PRP (Proline Rich Protein)	Pellicle precursors	5-30
Staherin	Inhibition of calcium precipitation	8
Histatin	Antimicrobial effects against Candida albicans	3-5
α-amylase	Starch degradation	54-57
Glucosyltransferases	Bacterial enzymes (glycan synthesizing)	140
Carbonic anhydrase VI	Neutralizes acids	42
Lysozyme	Antibacterial effect (lysis of bacterial wall)	14
Lactoferrin	Iron-binding glycoprotein, antibacterial properties	80
Mucin MG1	Lubricant action	>1000
Mucin MG2	Lubricant action	200-250
Cystatin SA, SN	Antibacterial and antiviral properties	9
Albumin	Regulation of colloid-osmotic pressure	69
sIgA, IgM, IgG	Immune response, inhibition of bacterial adhesion	60/90
Complement 3/3s	Activation of the complement system	210 (80 and 130)
Fibrinogen	Factor I of blood coagulation	340
Fibronectin	Structural protein	2225
Calgranulin C	Calcium binding	13
Cytokeratine 13, 15	Cellular protein	40-67
Salivary agglutinin	Agglutination of oral bacteria	300-400
Salivary amylase is found in the pellicle in two isoforms: a glycosylated isoform that is more abundant in the pellicle than the unglycosylated form.		

Enzymes such as salivary amylase, lysozyme, and bacterial glucosyltransferase, which are "trapped" within the pellicle layers, retain their enzymatic activity.

Serum components, fibrinogen, fibronectin, albumin, and IgG, are also detected in the dental pellicle. These proteins are more abundant in the pellicle formed on the gingival third of the tooth than in the pellicle formed on the incisal third. In individuals with healthy gingiva, significantly higher amounts of serum proteins (albumin, fibrinogen, and fibronectin) are present in the gingivally localized layers of the dental pellicle compared to those localized incisally. The increased amount of gingival fluid during inflammatory changes in the gingiva causes alterations in the protein composition of the dental pellicle. Specifically, the amount of serum proteins in the pellicle of the incisal third of the tooth increases. These findings once again confirm the notion that the availability of local salivary biopolymers influences both the formation and composition of the dental pellicle.

Analyses of pellicle formed under in vitro and in vivo conditions revealed certain differences. The pellicle formed in vitro contained a higher proportion of intact salivary proteins compared to that formed in vivo. This indicates that salivary proteins constituting the dental pellicle are subjected to extensive proteolytic processes within the oral cavity.

Intact proteins present in the dental pellicle formed under in vivo conditions include histatin 1, cystatin SN, statherin, lysozyme, albumin, amylase, cytokeratins 13 and 15, and calgranulin B. The presence of calgranulin B has been confirmed in both saliva and gingival fluid. The presence of cytokeratins in the dental pellicle indicates that the oral epithelium is a source of salivary proteins involved in the formation of the dental pellicle.

Modern immunological studies have confirmed that biomolecules integral to the dental pellicle include mucin type 1 (MG1), proline-rich proteins (PRP), statherin, histatin 1, albumin, amylase, secretory IgA, IgG, IgM, lactoferrin, lysozyme, and salivary carbonic anhydrase II.

Carbohydrates

The dental pellicle contains several different sugars, with glucose being the most abundant. Approximately half of the total sugar content in the pellicle is glucose. Other sugars present include galactose, mannose, fucose, glucosamines, and galactosamines. The origin of glucose in the dental pellicle is unclear but is most likely due to the direct adsorption of glucose polymers (dextran, i.e., glycans) originating from bacteria. The enzymes glucosyltransferases, adsorbed in the pellicle, participate in the synthesis of glycans, which in turn serve as a source of glucose for the dental pellicle. Also, glycolipids, which are the main representatives of lipids in the pellicle, are a possible source of glucose, since the glycoproteins constituting glycolipids contain only glucose.

Lipids

Lipids represent a significant constituent of the dental pellicle, comprising approximately 22–23% of its total dry mass. The most prevalent lipid components of the dental pellicle include the following:

1. Neutral lipids, predominantly composed of free fatty acids, triglycerides, cholesterol, and cholesterol esters;
2. Phospholipids, characterized by a high concentration of phosphatidylethanolamine, sphingomyelin, and phosphatidylcholine;
3. Glycolipids, which include both neutral and sulfated glyceroglycolipids.

It has been established that the lipid composition of the dental pellicle varies among individuals, which correlates with differences in caries activity. This variability is attributed to the ability of certain lipid classes to impede the diffusion of lactic acid through the pellicle layers, thereby providing a protective effect on the enamel surface. The ability of phospholipids to change the physicochemical properties of the pellicle, making it more resistant to the action of acids, is due to the interaction of this class of lipids with mucins.

Functions of the dental pellicle

The dental pellicle holds significant physiological and pathophysiological importance for all interactions between the tooth and its surrounding environment (saliva). It participates in several key processes within the oral cavity, including remineralization and demineralization, lubrication of the tooth surface, and bacterial aggregation.

Lubrication of the tooth surface

The dental pellicle exerts a lubricating effect on the tooth surface, facilitating both mastication and speech. This lubricating effect is attributed to salivary glycoproteins—mucin type 1, mucin type 2, and statherin—which are adsorbed within the layers of the dental pellicle. The high-molecular-weight mucin MG1 plays a particularly important role in this lubricating function. The dental pellicle reduces friction between opposing teeth and between teeth and the oral mucosa. Its presence between hard tooth surfaces has been shown to decrease the coefficient of friction by a factor of 20.

Dental pellicle as semipermeable membrane

The dental pellicle functions as a protective barrier that inhibits demineralization and promotes remineralization of the tooth enamel. Studies on in vitro-formed pellicles have demonstrated their selective permeability, enabling regulation of mineralization and demineralization processes at the enamel surface. This selective permeability allows the pellicle to modulate the diffusion of acids as well as the transport of calcium and phosphate ions into and out of the enamel. However, the precise mechanisms underlying the regulation of ion diffusion between the enamel surface and the oral environment remain not clearly elucidated.

Research has shown that open structures are present in the layers of the pellicle that allow the exchange of ions on the tooth surface. According to these indications, the pellicle is a medium through which fluorides, calcium and phosphates are delivered, during the remineralization process. The pellicle also delays or reduces the possibility of demineralization.

After experimental exposure of the pellicle to the action of 0.1% citric acid (period of 300 seconds), an uninterrupted continuity of the basal layers of

the dental pellicle is observed and no damage to the tooth enamel is observed. This data shows that the pellicle is partially resistant to the action of acids, and to a certain extent it can act as a diffusion barrier (Figure 10.3).

Notable variations in the ultrastructure, thickness, and composition of the dental pellicle are observed depending on the specific tooth surface on which it forms. The degree of protection against enamel erosion is closely associated with the pellicle's thickness.

For the protective role of the pellicle, not only the thickness is important, but also its composition. Salivary carbonic anhydrase VI is present in the dental pellicle and it shows enzymatic activity in the pellicle itself. This enzyme catalyzes the reversible hydration of carbon dioxide via the reaction: $CO_2 + H_2O \rightleftharpoons HCO_3^- + H^+$. When immobilized in the pellicle, salivary carbonic anhydrase VI facilitates the rapid neutralization of acids, thereby enhancing the pellicle's ability to protect enamel surfaces from acid-induced demineralization.

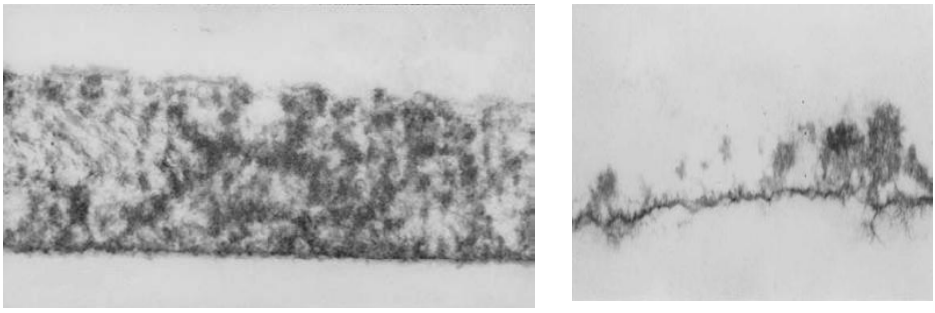


Figure 10.3: *Dental pellicle layers formed within 120 minutes (left), pellicle remnants (right) after treatment with 0.1% citric acid solution for 60 seconds*

Homeostasis of minerals

The dental pellicle modulates mineral precipitation processes on the tooth enamel surface. Saliva is supersaturated with calcium and phosphate ions, which is essential for the remineralization of demineralized tooth surfaces. Without the dental pellicle, calcium-phosphate salts would deposit continuously on the tooth surface. Acidic proline-rich proteins (PRPs), present in the pellicle, exhibit a high affinity for hydroxyapatite. These salivary proteins act as inhibitors of calcium salt precipitation on the tooth enamel surface, thereby maintaining mineral homeostasis between saliva and tooth enamel.

Modulation of bacterial adherence

The dental pellicle demonstrates selectivity in bacterial adherence and plays a role in the initial stages of dental bacterial biofilm formation. Its components function as receptors for bacteria. Salivary and bacterial enzymes alter the functional properties of the dental pellicle, making it a suitable substrate for bacterial adhesion.

11

BIOCHEMICAL CHARACTERISTICS OF THE BIOFILM

Biochemical characteristics of biofilm formation

Protein matrix of the biofilm

Adhesive properties of the protein matrix

Extracellular polysaccharides

Conditions for the synthesis of dextran and levan

Significance of extracellular polysaccharides

Metabolic activity of bacteria in the biofilm

Biochemical processes of bacteria in the biofilm

Synthesis and breakdown of glycogen (glycogenogenesis and glycogenolysis)
Glucose catabolism (glycolysis)
Lipogenesis

Urea catabolism

Synthesis of amino acids

Synthesis of toxic amines

pH CHANGES IN THE BIOFILM ENVIRONMENT

Classic concept of pH change in biofilm

Immature biofilm

Mature biofilm

A concept of pH change in biofilm based on the composition of its metabolic products

Decrease in plaque pH values

BIOCHEMICAL CHARACTERISTICS OF BIOFILM FORMATION

In contemporary scientific and professional literature, the term biofilm is increasingly being substituted with the term dental plaque. Biofilm denotes the acquired organic–bacterial deposit on the tooth surface. Simultaneously, biofilm constitutes a complex microbial community derived from the oral microflora. Biofilm is composed of:

- acquired dental pellicle
- bacteria from the oral flora
- matrix (proteins and polysaccharides)

Of the total biofilm content, 70–80% consists of bacteria, which is why this acquired deposit on the teeth is still referred to as bacterial biofilm. A single milligram of biofilm contains 10^8 different bacterial species originating from the oral flora.

The intercellular matrix is located among the bacteria within the biofilm and constitutes approximately 25% of its total mass. The intercellular matrix is composed of:

- salivary glycoproteins
- metabolic products of bacteria
- specific extracellular polysaccharides (synthesized by bacteria).

The plaque (biofilm) matrix serves as a substrate enabling bacterial cohesion and their adhesion to the enamel surface of the tooth. In addition, the intercellular matrix contains numerous harmful products released by bacteria, which induce inflammatory changes in the gingival tissue and damage the hard dental structures. The biofilm matrix also contains inorganic components: ions of Ca, phosphate, Mg, K, and Na. These components play a key role in the mineralization of the biofilm and the development of a hard deposit on the teeth — calculus.

In order to create the biofilm, bacteria need:

- to relate to the acquired dental pellicle
- to multiply
- to connect with each other

Bacteria reach the dental pellicle passively, thanks to the saliva flow and the constant contact of the teeth with the saliva.

From a biochemical point of view, three stages of biofilm creation are distinguished:

1. initial phase;
2. aggregation and accumulation phase; and
3. stage of recomposition or maturation;

A predominant process in **the initial stage** of plaque formation is the interaction between oral flora bacteria and the acquired dental pellicle. Glycoproteins form part of the bacterial cell wall, conferring a negative charge to the bacterial cell. Anionic residues (COO^-) of these glycoproteins extend outward, maintaining the bacterium's negative surface charge. The dental pellicle also contains various glycoproteins with similar anionic residues (COO^-). Consequently, direct adhesion of bacteria to the dental pellicle is not possible. Calcium ions (Ca^{2+}) present in saliva play a crucial role in the adhesion of the initial bacteria ("pioneers") to the dental pellicle. Due to their divalent positive charge, one valence binds to the (COO^-) groups of glycoproteins in the bacterial cell wall, while the other valence binds to the (COO^-) groups of glycoproteins within the dental pellicle. In this manner, electrostatic interactions between the anionic residues (COO^-) of pellicle glycoproteins and bacterial wall glycoproteins, mediated by calcium ions, facilitate the attachment of the first "pioneer" bacteria to the dental pellicle (Figure 11.1).

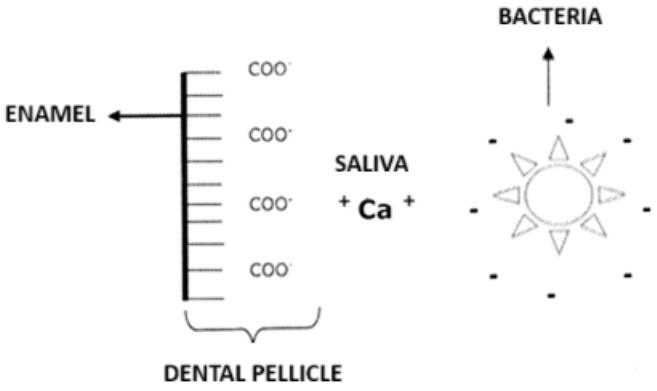


Figure 11.1: *Role of salivary Ca^{2+} ion in binding of the "pioneer" bacteria to the dental pellicle*

Hydrophobic interaction represents a secondary mechanism for bacterial adhesion to the dental pellicle (Figure 11.2). This interaction relies on the structural complementarity between hydrophobic molecules on the dental pellicle and those on the bacterial surface. Although this type of bacterial binding to the dental pellicle is not yet fully elucidated, it has been demonstrated that certain bacteria possess hydrophobic surface structures. One such structure is lipoteichoic acid, consisting of a hydrophilic glycerophosphate polymer and a nonpolar fatty acid tail, which provides an extensive hydrophobic surface.

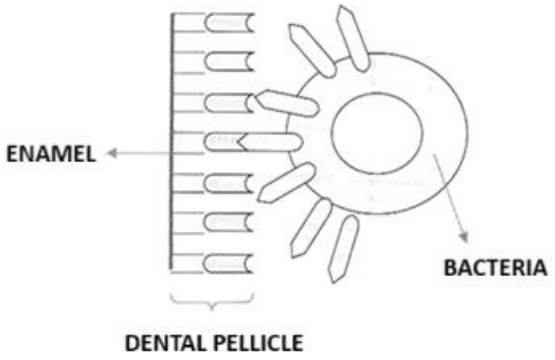


Figure 11.2: *Binding of bacteria by means of a hydrophobic interaction, which is based on a structural match between certain hydrophobic molecules of the dental pellicle and the same such molecules of the bacteria*

The electrostatic and hydrophobic bonds formed between bacteria and the dental pellicle are unstable and reversible. Apart from this type of connections, bacteria also connect to the dental pellicle by means of stable or irreversible connections.

Recent studies indicate that bacteria possess specific molecules (binding sites) capable of interacting with particular receptors in the dental pellicle. Bacterial surface molecules that can attach to these specific pellicle receptors are termed adhesins. Numerous organic components of the dental pellicle can function as bacterial binding receptors. Such components include proline-rich proteins, statherin, amylase, lysozyme, and immunoglobulins. There is a defined spatial complementarity between bacterial adhesins and the receptors in the dental pellicle (Figure 11.3).

This way of connecting bacteria is not only significant for their adherence to the surface of the tooth, but also for their adherence to the oral mucosa. Epithelial cells of the oral mucosa possess specific receptors for binding to bacterial adhesins. Those receptors of epithelial cells are: sialic acid, galactosyl residues, collagen fibers and fibronectin.

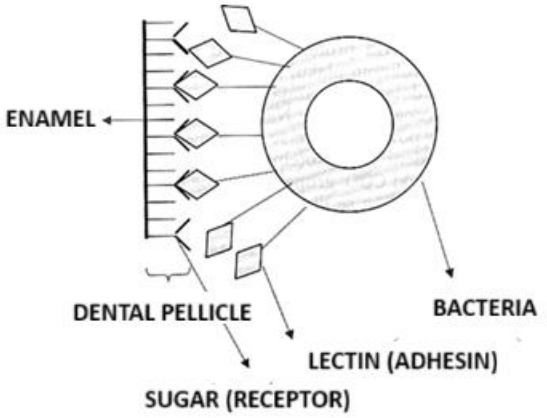


Figure 11.3: *Binding of bacteria to the dental pellicle by means of stable irreversible bonds between lectins and specific receptors (sugars)*

Lectins, which are specific proteins, function as adhesins on the bacterial cell surface. These lectins recognize particular sugar residues (receptors) within the dental pellicle, corresponding to the carbohydrate portions of pellicle glycoproteins. Bacterial lectins can attach to specific sugar residues on dental pellicle glycoproteins, facilitating bacterial adhesion to the pellicle. Some bacteria target galactosyl residues, whereas others bind to sialic acid residues. This binding mechanism is crucial for bacterial adherence to the dental pellicle and for bacterial interconnection (aggregation).

Following the initial adhesion of "pioneer" bacteria to the dental pellicle, the second phase of biofilm formation, **the aggregation and accumulation phase,** commences. During this phase, bacteria proliferate and establish interconnections within the biofilm. The first colonizing bacteria metabolize the carbohydrate components of glycoproteins from the dental pellicle to obtain the energy required for survival and multiplication. Removal of the carbohydrate portion of the glycoproteins leaves only the "bare" proteins, which constitute the dental plaque matrix.

Protein matrix of the biofilm

The protein matrix constitutes a major component of dental plaque, although its origin remains debated. The most widely accepted view is that the protein matrix derives from modified salivary glycoproteins, specifically those

forming the dental pellicle. Salivary glycoproteins undergo modifications, including loss of their carbohydrate moieties, due to the activity of extracellular enzymes from plaque bacteria. Carbohydrate removal occurs in response to the bacteria's high demand for sugars. In addition to carbohydrate loss, polypeptide chains are degraded to the level of peptides.

By biochemical analysis of the protein matrix, it was determined that, unlike saliva, it does not contain sialic acid. Sialomucin is included in the composition of the dental pellicle, but not in the protein matrix of the dental plaque, since the carbohydrate part of the sialomucin is catabolized under the action of enzymes that release the bacteria from the plaque.

One of the most active enzymes secreted by bacteria into the extracellular environment is sialidase. This enzyme specifically targets sialic acid residues within the macromolecule sialomucin. Through its action, the sialic acid residues are cleaved, resulting in the deposition of sialomucin. This process leads to irreversible denaturation of sialomucin and loss of its biological functions.

Native sialomucin (present in saliva) exhibits a very low isoelectric point of pK = 2.6. This property prevents its deposition even at the lowest physiological salivary pH values. Marked acidification and a substantial local pH drop (e.g., to pH 2) are uncommon under normal conditions, but if they occur, such as from gastric reflux, sialomucin re-precipitation may take place. Deposition continues until the local pH is restored by the buffering action of saliva.

Consistent with the known properties of sialomucin, it is unlikely that local pH changes within the plaque, resulting from acidic metabolites, will induce its deposition. Conversely, bacterial sialidase promotes the deposition and denaturation of sialomucin present in the dental pellicle or delivered to the plaque via saliva.

The protein matrix is important for the aggregation of numerous and diverse bacteria into colony-like structures. This arrangement brings bacteria into close proximity, highlighting the adhesive properties of the protein matrix. As a result, a large number of bacteria accumulate within a relatively confined space, leading to an increase in acidic metabolites and toxic products within the plaque. These substances, in turn, exert a dual detrimental effect:

- harmful effects on the solid dental substances;
- inflammatory changes of the gingiva and supporting tissues of the tooth.

During the initial stages of plaque formation, while still immature, the

protein matrix has formed, but the spaces between bacteria remain sufficiently large to permit fluid flow and removal of released metabolites. As the plaque matures and extracellular polysaccharides are produced, the microspaces between bacteria narrow to the extent that they restrict diffusion of both fluid and metabolites.

Adhesive properties of the protein matrix

Following the removal of sialic acid from glycoprotein molecules by sialidase, the glycoproteins lose key properties, including extensibility, hydration, and solubility stability. Once the carbohydrate components are removed, only the “bare” peptide chains remain. These peptide chains possess adhesive properties and facilitate the connection of bacteria within the dental plaque. In this manner, the protein matrix performs its primary function: aggregating a large number of bacteria within a relatively confined space. (Figure 11.4 and 11.5).

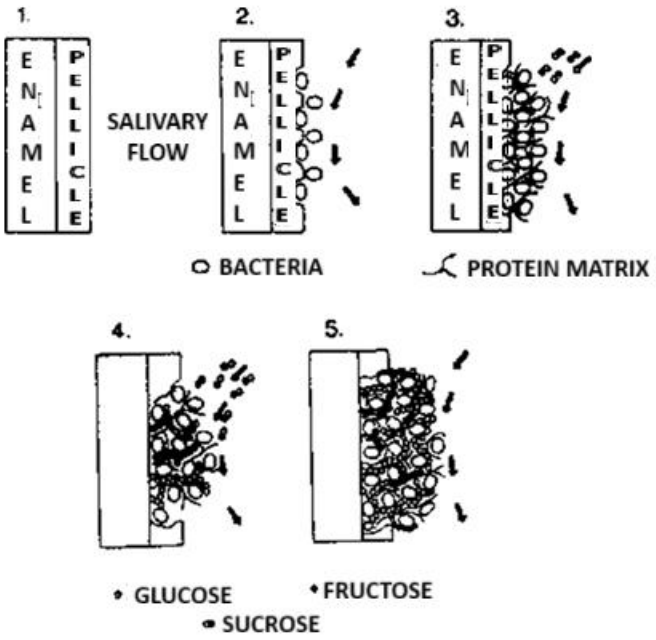


Figure 11.4: *Stages of plaque formation: (1) enamel with the dental pellicle in contact with the saliva; (2) initiation of colonization and changes in pellicle composition; (3) formation of the protein matrix; (4) "maturation" of the plaque with the inclusion of sucrose; (5) fully formed "mature" plaque with the presence*

of extracellular polysaccharides.

The adhesive effect of peptide chains can be explained as follows: bacterial cell walls contain glycoproteins with free dissociated carboxyl –OOH groups, which contribute to mutual bacterial repulsion. Peptide chains possess both amino $–NH_2$ and carboxyl –COOH terminal groups, allowing them to insert between adjacent bacteria so that the NH_2 end interacts with the COOH group of the bacterial wall. Prior to this interaction, the NH_2 group of the peptide chain reacts with water, forming a base that dissociates into an NH_3^+ cation and an OH group according to the following reaction:

$$\mathbf{P\text{-}\ NH_2 + H_2O \rightarrow P\text{-}NH_3OH \rightarrow P\text{-}NH_3 + OH\ (P{=}peptide)}$$

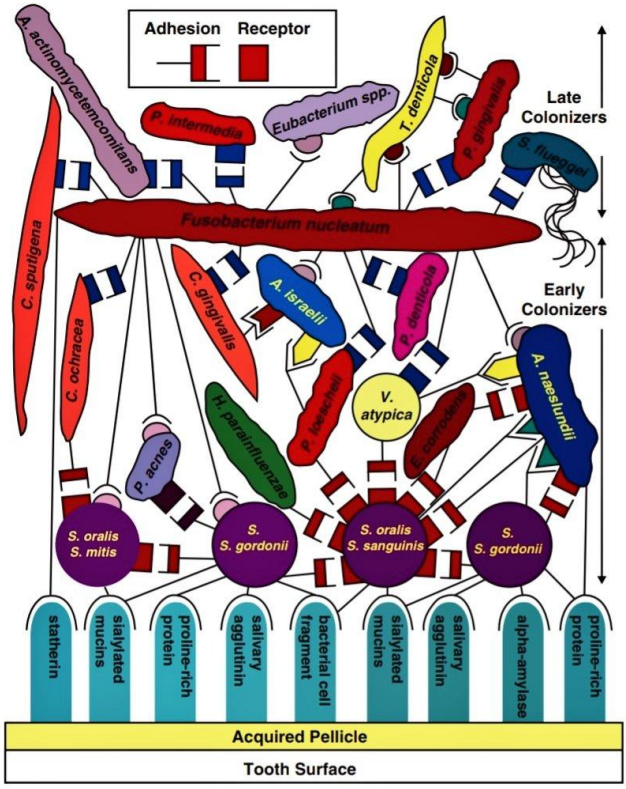


Figure 11.5: *Schematic representation of the binding of different bacteria to the tooth surface, their interaction and formation of the biofilm*

The positively charged amino-terminal end of the peptide facilitates the formation of an ionic bond with a dissociated COOH group on the bacterial wall. The opposite end of the peptide, being carboxy-terminal in an intact peptide

chain, cannot react with the same bacterial COOH group. However, as the dental plaque fluid is rich in electrolytes, particularly calcium, a calcium ion can bridge the two carboxyl groups, thereby linking adjacent bacteria (Figure 11.6). If the peptide is fragmented and possesses free NH_2 groups at both ends, the calcium-mediated binding is not required.

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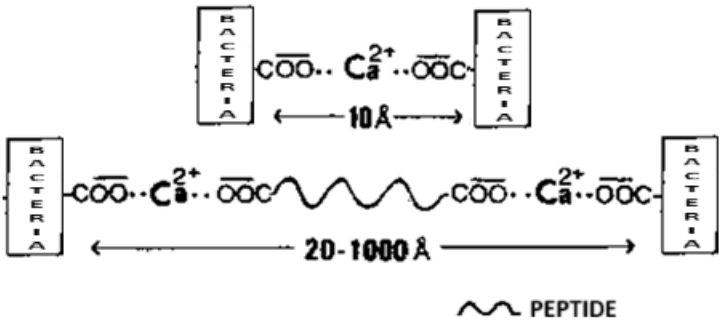

Figure 11.6: *The binding role of Ca, that is, the connection of this ion and the peptide fragment former during the creation of the protein matrix in the dental plaque.*

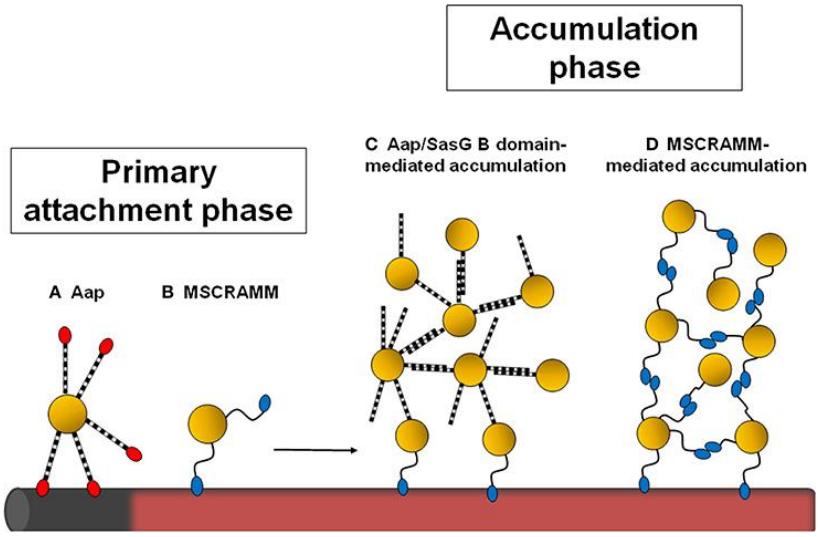

Figure 11.7: *Initial bacterial colonization and biofilm accumulation enabled by peptides of the protein matrix*

Bacterial aggregation in dental plaque is also facilitated by an immunochemical mechanism. Salivary immunoglobulins contribute to plaque bacterial adhesion, as they function as antibodies. Antibody synthesis occurs in response to antigenic stimulation, with bacterial wall components serving as antigens. Consequently, individual salivary immunoglobulins (predominantly IgA) can mediate binding between at least two bacteria through antigen–antibody interactions. Secretory IgA, being a dimer, possesses four antigen-binding sites, allowing the linkage of three to four bacteria in close proximity. Immunoglobulin M, which can form a pentamer, exhibits comparable binding capabilities.

The first created colonies of bacteria, over time, begin to synthesize extracellular polysaccharides, which also become part of the biofilm matrix.

The beginning of the synthesis of extracellular polysaccharides also marks the beginning of the third phase of the creation of the biofilm, that is, the phase of recomposition or maturation.

Extracellular polysaccharides

Extracellular polysaccharides constitute a distinct component of dental plaque. Their presence indicates the "maturation" of the plaque. The emergence of these polysaccharides imparts new properties and clinical significance to dental plaque, as they enable potential damage to the teeth, gingiva, and supporting structures. Extracellular polysaccharides include two types: dextran and levan.

Dextran is a polysaccharide formed by the polymerization of numerous glucose molecules. Since glucose is also referred to as dextrose, the polysaccharide is logically named dextran. Dextran consists of glucose units linked primarily by α-1,6 glycosidic bond. The dextran molecule is branched, with side chains connected by secondary type of α-1,4 glycosidic bond, and additional tertiary chains linked via α-1,3 bond. (Figure 11.8).

Levan is the second major extracellular polysaccharide in dental plaque. It is composed of multiple fructose units. As fructose is also known as levulose, the polysaccharide is appropriately named levan. Levan consists of an unbranched chain in which the fructose units are linked by β-2,6 glycosidic bonds. (Figure 11.8).

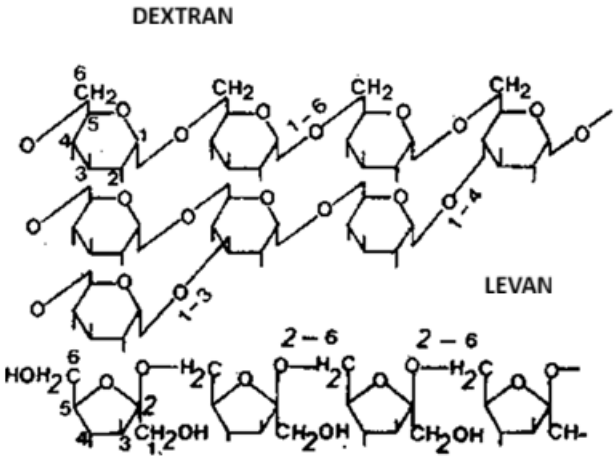

Figure 11.8: *Structure of the extracellular polysaccharides dextran and levan. The basic chain of dextran is branched, while that of levan is not.*

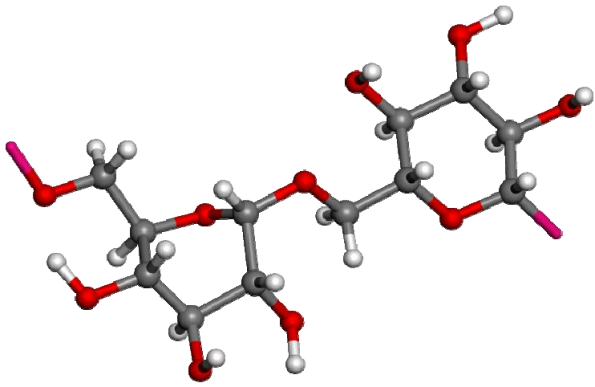

Figure 11.9: *Branched chain of dextran*

The synthesis of dextran and levan occurs within dental plaque and represents an anabolic process that requires adequate sucrose availability. Sucrose, a sugar commonly consumed in the human diet, is a disaccharide composed of glucose and fructose. Hydrolysis of sucrose provides the essential subunits for dextran and levan synthesis. Additionally, sucrose readily dissolves in water, is electrically neutral, and has a low molecular weight, allowing efficient diffusion into the plaque microenvironment. These properties make sucrose the preferred substrate for rapid and effective synthesis of the extracellular polysaccharides dextran and levan.

In order to ensure rapid and massive formation of mature dental plaque, it is necessary to meet two basic principles:

- Poor oral hygiene and
- Using a large amount of the sugar sucrose.

The synthesis of extracellular polysaccharides is mediated by specific bacterial enzymes, namely dextran sucrase and levan sucrase. These enzymes may be free in the plaque fluid or incorporated with the bacterial cell wall. They belong to the glucosyl- or fructosyl-transferase class, catalyzing the transfer of glucose or fructose residues and their polymerization into dextran or levan through sucrose hydrolysis.

The reactions of the breakdown of the sugar sucrose and the further synthesis of the extracellular polysaccharides are two interdependent reactions that can be represented in the following way:

$$n\ \text{Sucrose} + n\ H_2O \xrightarrow{\text{Dextran Sucrose}} \text{Dextran} + n\ \text{Fructose}$$

From the depicted reaction, sucrose molecules are hydrolytically cleaved by dextran sucrase. This enzyme also facilitates the transfer of glucosyl residues and their incorporation into the extracellular polysaccharide dextran. A substantial number of fructose residues remain free, which logically leads to a subsequent reaction in which these fructosyl residues are utilized during sucrose decomposition.

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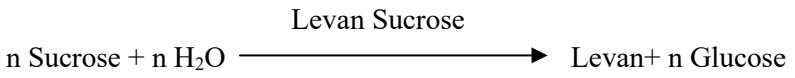
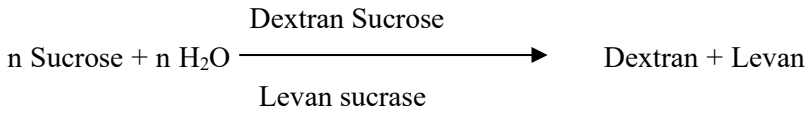


The mentioned enzymatic reactions, in which two different enzymes use the same substrate (sucrose), can be united in one reaction. This is quite logical since the synthesis of both extracellular polysaccharides takes place simultaneously.



Conditions for the synthesis of dextran and levan

The synthesis of these extracellular polysaccharides, which are crucial for plaque maturation, exhibits specific structural characteristics. Dextran closely resembles glycogen, the animal polysaccharide, in its overall structure. Like dextran (Figure 11.9), glycogen has a branched main chain, although the main-chain linkages are α-1,4 glycosidic bonds. Secondary side chains are attached to the main chain via α-1,6 glycosidic bonds. Tertiary chains extend from the secondary chains, also connected through α-1,6 glycosidic bonds.

It is obvious that there is a great structural similarity between these two polysaccharides. But there are significant differences in their synthesis.

To initiate glycogen synthesis in the liver or muscles, activation energy is required because glucose is chemically inert. This activation energy is supplied by ATP, an energy-rich compound with universal roles in both anabolic and catabolic metabolic processes. Enzymes such as hexokinase or glucokinase catalyze the enzymatic activation of glucose. For each glucose molecule activated, one mole of ATP is consumed, producing an activated glucose-6-phosphate molecule.

The synthesis of extracellular polysaccharides occurs in the extracellular (extrabacterial) environment, where ATP is absent. Consequently, glucose and fructose molecules cannot undergo activation. Dextran and levan synthesis is an endoenergetic process, requiring 4 kcal/mol for dextran polymerization and 2 kcal/mol for levan. Thus, an additional 6 kcal/mol of energy must be supplied for

each incorporated glucose or fructose unit.

That energy (6 kcal/mol) is provided by the hydrolysis of sucrose, because the hydrolytic decomposition of this disaccharide releases a total of 7 kcal/mol.

$$\text{Sucrose} + H_2O \xrightarrow{\text{Hydrolysis}} \text{Glucose} + \text{Fructose} - 7 \text{ kcal/mol}$$

An excess of 1 kcal/mol does not represent an obstacle for the synthesis of these extracellular polysaccharides, but, on the contrary, facilitates the process of obtaining dextran and levan.

Importance of extracellular polysaccharides

Dextran is a poorly soluble polysaccharide that contributes to an increase in plaque volume. Under optimal conditions for extracellular polysaccharide synthesis, plaque exhibits a dense, striated appearance due to dextran, which is the predominant polysaccharide.

Dextran possesses a high adsorption affinity for hydroxyapatite, enhancing bacterial attachment to the tooth surface. This results in a form of plaque "cementation," which at this stage can only be removed mechanically (toothpaste and brush). The adsorption capacity of dextran is reduced in the presence of phosphates.

Dextran exhibits the ability to agglutinate certain bacteria, such as *Streptococcus mutans*, which is highly relevant for dental plaque formation. With dextran present in the plaque, diffusion is markedly reduced due to narrowing of the microspaces between bacteria. Consequently, the diffusion of bacterial metabolites, movement of local fluids, and penetration of salivary bicarbonates, which are critical for buffering both the oral cavity and the plaque microenvironment, are impeded.

The reduced ability to diffuse with the aid of dextran is explained in two ways:

1. Physical - narrowing of microspaces between bacteria;
2. Electrochemical - with the help of phosphates, which are ester-bound to the dextran itself.

Within the plaque, the enzyme dextranase is present, enabling the hydrolytic degradation of dextran. This indicates that dextran breakdown and removal are possible; however, the enzyme's activity is minimal, and complete removal of dextran does not occur.

Levan is a less significant extracellular polysaccharide in dental plaque. It does not play a permanent role, as it is easily synthesized and likewise readily degraded by the specific enzyme levanase. Through levanase, bacteria hydrolytically cleave levan into fructose molecules, which can then be utilized for their metabolic needs under conditions of limited sugar availability.

In addition to extracellular polysaccharides, bacteria can synthesize intracellular (intrabacterial) polysaccharides. Based on its α-1,4 glycosidic bonds and glucose subunits, this macromolecule closely resembles glycogen. The presence of such bacteria in dental plaque indicates the occurrence of caries-active species. Under conditions of limited dietary sugar intake, these bacteria can mobilize intrabacterial polysaccharides, breaking them down into glucose units. Subsequent glucose metabolism releases energy for bacterial activity and produces lactic acid, which is excreted into the extracellular environment, lowering the local pH. Studies have shown that low concentrations of fluoride in plaque effectively inhibit intrabacterial polysaccharide synthesis.

Based on dextran and levan content, as well as bacterial number and type, two biofilm categories are recognized: immature and mature. Immature biofilm is a thin, visually imperceptible plaque on teeth that forms within 24 hours after brushing. It contains few bacteria and proteins, and extracellular polysaccharide formation has not yet begun. Because it lacks dextran and levan, this biofilm is thin, porous, and contains numerous interstices. Its porosity allows diffusion of metabolites from the biofilm into saliva and vice versa. During bacterial metabolic activity, various metabolites, both acidic and basic, are released, which can be harmful to tooth enamel and the supporting structures.

The most significant acidic metabolite is lactic acid (lactate), produced via anaerobic glycolysis, which provides energy for the bacteria. In an immature, porous biofilm, lactate diffuses into the saliva and does not accumulate within the biofilm. Salivary diffusion into the biofilm also occurs, allowing bicarbonates to exert an alkalizing effect. Due to these diffusion properties, metabolites released by bacteria do not accumulate long-term, and the biofilm pH remains similar to that of saliva (6.1–7.8). At this pH, hydroxyapatite crystals remain stable and demineralization does not occur. The critical pH in the biofilm, at which demineralization begins, is 5.5.

Mature biofilm develops from the young, immature biofilm when bacteria begin synthesizing extracellular polysaccharides, dextran and levan, typically 4–10 days after biofilm formation begins. The bacterial population increases markedly, and anaerobic microorganisms appear. As extracellular polysaccharide synthesis intensifies, the microspaces between bacteria become filled, making the mature biofilm nearly non-porous. Diffusion of harmful bacterial metabolites from the biofilm is reduced, and the rinsing effect of saliva is hindered, limiting the penetration of salivary bicarbonates into the biofilm microenvironment. Consequently, bacterial metabolites, both acidic and basic, accumulate in the mature, non-porous biofilm, while the buffering capacity of saliva is significantly diminished. The accumulation of acidic metabolites, which causes a substantial and prolonged decrease in biofilm pH, is particularly detrimental to tooth enamel. When the pH in the mature biofilm reaches the critical value of 5.5, hydroxyapatite crystals on the enamel begin to dissolve, initiating demineralization and the development of dental caries.

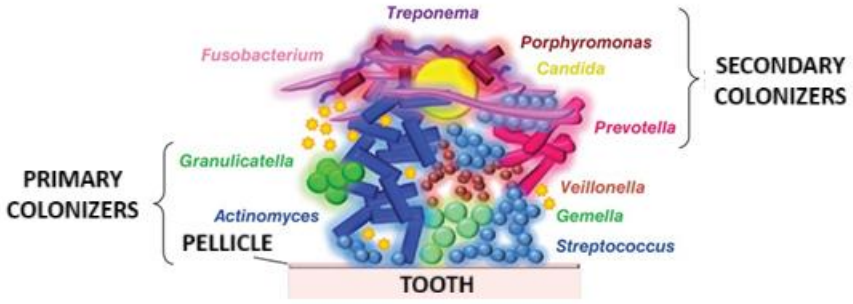


Figure 11.10: *Plaque Formation Model – Primary colonizers attach to components of the acquired pellicle on the tooth surface and to each other through co-adhesion and co-aggregation, forming a sublayer for interaction with secondary colonizers. As plaque matures, the synthesis of extracellular polysaccharides provides structural stability and facilitates molecular exchange. Nutritional adaptation, intermicrobial signaling (yellow stars in the image), and beneficial or antagonistic interactions promote the development of distinct "microbial communities" within the plaque, supporting optimized metabolic networks and protective functions. Ultimately, a microbiological community is established that exists in equilibrium with the host.*

Metabolic activity of bacteria in the biofilm

A wide range of diverse and intensive metabolic processes occur within the bacterial biofilm. The bacteria in the biofilm are heterotrophic microorganisms, meaning they derive the energy substrates required for their metabolism from the local environment. These substrates include biopolymers originating from saliva and gingival fluid, as well as nutrients introduced into the oral cavity. Nutrients represent the primary energy source for the bacteria. To incorporate these biomolecules into appropriate energy-yielding pathways, they must first be broken down into their basic components. This breakdown is enabled by specific enzymes produced by the bacteria within the biofilm.

Considering the substantial requirement of biofilm bacteria for carbohydrate and nitrogen compounds, the breakdown of complex biomolecules is essential for their survival and proliferation. This is because biomolecules (polysaccharides, proteins, and lipids) cannot be transported into the bacterial environment either by diffusion or active transport.

Polysaccharides and disaccharides represent the most abundant biopolymers; therefore, their hydrolysis to the basic components—monosaccharides—is of critical importance for bacterial survival within the biofilm. The monosaccharides obtained during the degradation of polysaccharides and disaccharides are subsequently integrated into the respective biochemical pathways in bacterial cells, where the required energy is generated. Among carbohydrate-based biopolymers, bacteria are capable of degrading starch, hyaluronic acid, chondroitin sulfate, as well as disaccharides such as maltose, lactose, and sucrose.

Starch originates from nutrients that are ingested daily into the oral cavity. It is broken down under the action of the bacterial enzyme *β-amylase*, which breaks down starch into maltose, isomaltose and maltotriose. Maltose, ingested with food or obtained during the breakdown of starch, is broken down into glucose molecules under the action of the bacterial enzyme *maltase*.

Sucrose, which is ingested with food, is hydrolyzed into glucose and fructose molecules by the catalytic action of the bacterial enzyme *sucrase*.

Lactose, which is also ingested with food, is hydrolyzed into glucose and galactose molecules under the action of the bacterial enzyme *lactase*.

Hyaluronic acid is an essential component of the soft oral tissues

(gingiva), incorporated into proteoglycans that serve a binding function. Proteoglycans consist of proteins and a prosthetic group—hyaluronic acid. Hyaluronic acid is degraded into its basic components by the bacterial enzyme hyaluronidase. Through the action of this enzyme, the structural biochemical components of the gingiva are broken down, leading to the development of periodontal disease.

Chondroitin sulfate is a structural component of hard tissues, primarily bone and cartilage. Certain bacteria within the biofilm secrete the enzyme *chondroitin sulfatase*, which degrades chondroitin sulfate into its basic constituents. The breakdown of this structural component of the alveolar bone results in its destruction and resorption, processes characteristic of periodontal disease.

For monosaccharides obtained from the degradation of carbohydrate biopolymers to be utilized for energy production, they must first be transported into the bacterial cell. The principal mechanisms of simple sugar transport in bacteria are:

1. a specific, phosphoenolpyruvate-mediated system or the so-called phosphotransferase transport system (PEP-PTS).
2. nonspecific transport systems for different sugars.
3. glucose-permease transport system.

The most important transport mechanism is the phosphoenolpyruvate-dependent system, known as the phosphotransferase transport system (PEP-PTS). This is a highly specific system for the transport of monosaccharides in bacteria. It involves several enzymes and proteins localized in the cytoplasm and the bacterial cell membrane. The system functions through the transfer of a phosphate group from phosphoenolpyruvate (PEP), an intermediate of glycolysis, via two cytosolic proteins: HPr and Enzyme I (not sugar-specific). The phosphate group is then passed to the membrane-associated Enzyme II, which is strictly specific for certain sugars. Enzyme II catalyzes both the transport and phosphorylation of the sugar as it enters the bacterial cell. Thus, the phosphate group, originally derived from phosphoenolpyruvate (PEP), is transferred via HPr in the form of HPr-P to Enzyme II. This transport system is particularly specific for glucose uptake in bacterial cells (Figure 11.11).

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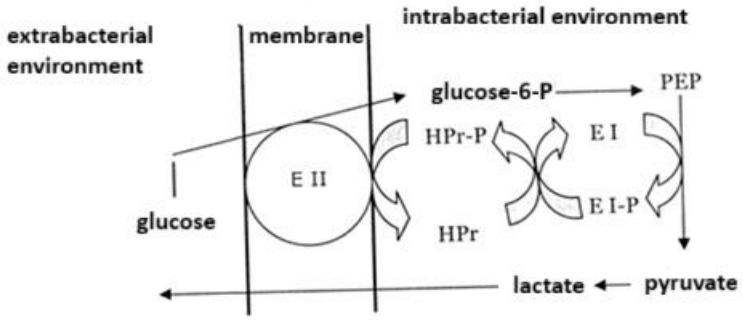


Figure 11.11: *Schematic representation of glucose transport in the bacterium using the PEP-PTS system*

The second mechanism for transporting simple sugars in bacteria is the non-specific transport system. This system can transport a variety of sugars. Its role in the metabolism of biofilm bacteria is not yet fully understood. It participates in the transport of end products from the extracellular breakdown of polysaccharides when the bacterial cell lacks sugars for energy production.

When sugar concentrations in the bacterial environment of the biofilm are high, the activity of the PEP-PTS transport system decreases. Under these conditions, sugars are transported into the bacterial cell via an ATP-dependent system involving the enzyme *glucose-permease*. In this scenario, the bacterial cell tends to synthesize glycogen due to the "surplus" of sugars.

Once monosaccharides are transported into the bacterial cell, they are utilized in catabolic or anabolic processes according to the metabolic requirements of the biofilm bacteria.

Proteins, like carbohydrates, are broken down into amino acids by specific bacterial enzymes, and these amino acids participate in the corresponding catabolic or anabolic pathways.

Proteins degraded by bacteria originate from saliva, ingested food, or the structural proteins of the soft and hard tissues of the tooth-supporting apparatus. Degradation of structural proteins in these tissues results in the destruction of the gingiva, periodontal fibers, and alveolar bone—pathological processes characteristic of periodontal diseases. Among the structural proteins of the oral

tissues, the most important are the supporting proteins, collagen and elastin. Collagen, found in the gingiva, dentin, cementum, alveolar bone, and periodontal fibers, is broken down by the bacterial enzyme *collagenase*. Elastin, present in soft oral tissues, is degraded by elastase. Bacterial enzymes that degrade proteins from saliva or ingested food belong to the group of *proteases.*

Lipids and phospholipids, which are part of the cell membrane, can be broken down under the action of bacterial enzymes *lipases* and *phospholipases* to their basic components.

Biofilm bacteria, according to their metabolic characteristics, are divided into two groups:

1. bacteria that break down nitrogenous compounds (proteins). They are also called proteolytic bacteria.
2. bacteria that break down carbohydrates. They are also called saccharolytic bacteria.

The first group of bacteria that decompose nitrogenous compounds releases basic metabolites (such as basic amino acids, ammonia, and toxic amines) into the biofilm microenvironment. The release of these basic metabolites increases the pH in the biofilm. While this does not harm the tooth, it can negatively affect the periodontal tissues. The second group of bacteria, which break down sugars, releases acidic metabolites, including lactate and other organic acids. The accumulation of these acidic metabolites lowers the pH in the biofilm, potentially leading to demineralization of the tooth enamel.

Biochemical processes of bacteria in biofilm

A large number of diverse metabolic processes occur in bacterial biofilms, many of which are similar to those in human cells. These include catabolic processes that release energy and anabolic processes for synthesizing organic compounds necessary for bacterial survival. The key metabolic processes in bacterial cells are:

Synthesis and breakdown of glycogen (glycogenogenesis and glycogenolysis)

Breakdown of glucose (glycolysis)

Tricarboxylic acid (TCA) cycle

Oxidative phosphorylation

Hexose monophosphate pathway

Lipogenesis

Urea catabolism

Amino acid catabolism

Synthesis of toxic amines

Amino acid synthesis

Purine and pyrimidine synthesis

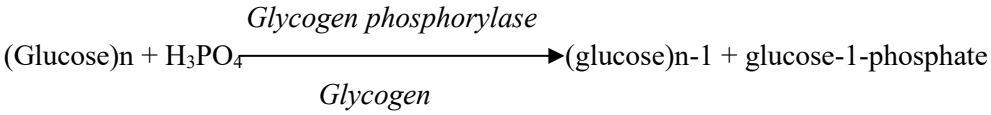
Protein synthesis

Synthesis and breakdown of glycogen (glycogenogenesis and glycogenolysis)

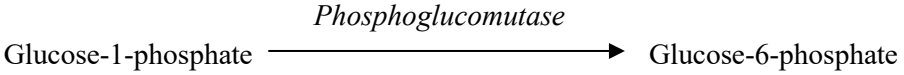
The processes of glycogenolysis and glycogenogenesis are antagonistic. In glycogenolysis, glycogen is broken down into glucose-1-phosphate molecules to meet the bacteria's energy needs. In glycogenogenesis, glycogen mass increases by incorporating new glucose molecules. To ensure these opposing processes occur efficiently, the enzymes required for one process are inhibited whenever the other process is active.

Glycogen serves as a glucose reserve in bacterial cells, similar to its role in human cells. When the bacterial cell lacks glucose as a primary energy source, glycogen is degraded through glycogenolysis, providing the bacterium with an endogenous supply of glucose. Glycogenolysis in bacteria proceeds in a manner very similar to that in human muscle tissue. The bacterial enzymes involved are identical to those regulating glycogenolysis in humans: *glycogen phosphorylase, phosphoglucomutase, trisaccharide transferase, and the debranching enzyme.* Like muscle cells, bacterial cells lack *glucose-6-phosphatase*; consequently, the resulting glucose-6-phosphate molecules enter glycolysis and are further metabolized to lactic acid.

The breakdown of glycogen is phosphorylytic, and for each released molecule of glucose, one molecule of orthophosphate is used.



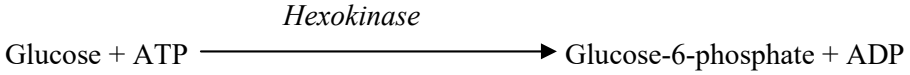
In order for glucose-1-phosphate to be used, it is converted into glucose-6-phosphate, with the help of the enzyme *phosphoglucomutase*.



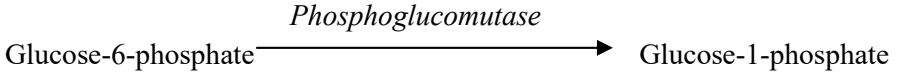
When all intracellular bacterial glycogen reserves are depleted and the bacterium still requires glucose, the extracellular polysaccharide levan is degraded. The bacterial enzyme *levanase* breaks down levan into fructose molecules, which are then used as an energy source.

When glucose is abundant in the bacterial biofilm, the bacterium initiates glycogen recovery and storage, i.e., glycogenogenesis. This biochemical process closely resembles glycogenogenesis in human cells.

For glycogenesis to commence in the bacterial cell, glucose from the extracellular environment must first be transported into the cell. Inside the bacterial cell, glucose is phosphorylated by the enzyme hexokinase, with each glucose molecule requiring one molecule of ATP (adenosine triphosphate).

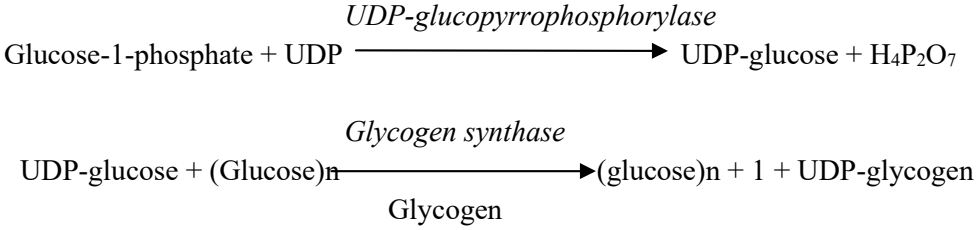


The resulting product, glucose-6-phosphate, indicates that glucogenesis can begin. Under the influence of the enzyme *phosphoglucomutase*, glucose-6-phosphate is transformed into glucose-1-phosphate.



Uridine triphosphate (UTP) is a coenzyme involved in glycogenesis, facilitating the transfer and incorporation of glucose into the glycogen molecule. This reaction produces uridine diphosphate (UDP-glucose) and pyrophosphate,

with the enzyme UDP-glucosopyrophosphorylase catalyzing the process.



Glucose catabolism (glycolysis)

Glycolysis is a fundamental catabolic process that breaks down glucose and other monosaccharides to provide energy for bacterial cells in the biofilm. Glucose in the biofilm bacteria is metabolized in two ways: oxidative (aerobic) and fermentative (anaerobic). These two pathways differ in metabolic steps, ATP production mechanisms, and final metabolic products. However, the initial stage of glucose breakdown is the same for both pathways, in which glucose is converted into two molecules of pyruvate. The subsequent metabolic fate of pyruvate depends on the bacterial type.

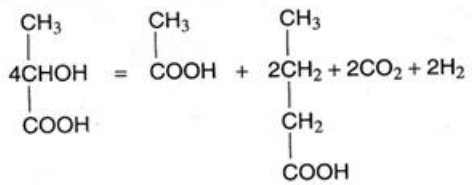
Oxidative glucose metabolism occurs in aerobic and facultatively anaerobic bacteria. Pyruvate, produced during the first stage of glycolysis, is converted into acetyl-CoA. The resulting acetyl-CoA enters the tricarboxylic acid cycle (TCA cycle, Krebs cycle, or citric acid cycle), where it is completely oxidized to CO_2 and H_2O. During the TCA cycle, reduced coenzymes NADH + H^+ and $FADH_2$ are generated, which are reoxidized via the respiratory chain, transferring protons and electrons to O_2. In bacteria, the respiratory chain is located in the cytoplasmic membrane, as they lack mitochondria. Molecular oxygen serves as the final acceptor of electrons and protons, while ATP is synthesized through oxidative phosphorylation.

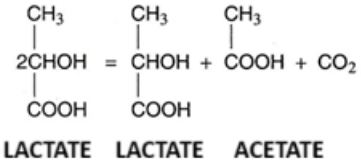
In obligate anaerobic bacteria, sugars are broken down via *fermentative metabolism.* In this process, sugars are converted into low-molecular-weight organic molecules, such as organic acids and alcohols. The respiratory chain is not involved, and oxygen does not act as the final electron and proton acceptor.

Energy (ATP) is obtained solely through substrate-level phosphorylation. In anaerobic bacteria within the biofilm, glycolysis concludes with the formation of lactate. Specifically, pyruvate produced in the first stage of glycolysis is reduced to lactate under the action of *lactate dehydrogenase*. This reaction also allows the reoxidation of NADH + H^+, which is generated during the oxidation of glyceraldehyde-3-phosphate in glycolysis. In this context, the reduced NADH + H^+ cannot transfer electrons and hydrogen atoms to a respiratory chain but instead transfers them to another substrate. That other substrate is pyruvate. Pyruvate is reduced by the catalytic action of the enzyme *lactate dehydrogenase*, producing lactate, which is the end product of anaerobic glycolysis. Lactate is released into the extrabacterial environment, i.e., the microenvironment of the biofilm. If conditions favor the accumulation of lactate in the biofilm, it will cause a significant decrease in the pH and create a predisposition for demineralization. Fluoride, when applied locally to the tooth, has been shown to inhibit glycolytic processes in the bacteria of the biofilm by inhibiting the enzyme *enolase*, which is involved in glycolysis. By inhibiting glycolysis, lactate production is reduced, preventing the decrease in pH in the biofilm. In this way, fluoride helps prevent demineralization of tooth enamel in an acidic environment. The preventive effect of fluoride against dental caries is based, in part, on this mechanism.

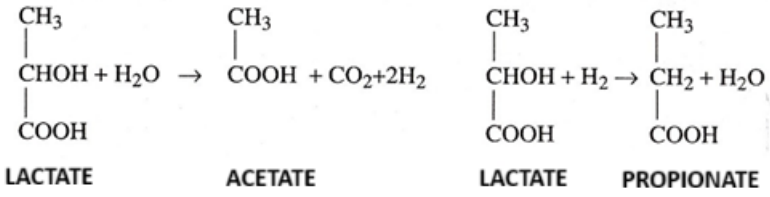
The process of anaerobic glycolysis in a bacterial cell is identical to glycolysis in human cells.

Some bacteria in the biofilm (Clostridium, Veillonella) continue the catabolitic process of glucose, breaking down lactate to acetate, butyrate and propionate.





The microorganism Veillonella lacks its own enzymes for glucose catabolism. It utilizes lactate produced by Streptococcus during glycolysis, converting it into acetate and propionate. Since acetate and propionate are weaker acids than lactate, Veillonella increases the local pH in the biofilm. Thus, this microorganism exerts a local buffering effect in dental plaque.



Lipogenesis

Lipogenesis is the process of lipid synthesis. Bacteria in the biofilm, like all other cells, contain lipids in their cell membranes and therefore need to synthesize complex organic compounds such as lipids.

Triglycerides are synthesized from glycerol and fatty acids. In bacterial cells, glycerol is derived from glucose, which is broken down into intermediate products via glycolysis. Activated glycerol, in the form of glycerol-3-phosphate, is obtained from these intermediates. Similarly, in human adipocytes, glycerol is produced from glucose intermediates for the purpose of lipid synthesis.

Fatty acids in bacteria are synthesized similarly to human cells, using the palmitic acid synthesis system (PASS). PASS enables the production of palmitic acid and other saturated fatty acids with fewer than 16 carbon atoms. The starting compounds for fatty acid synthesis are acetyl-CoA and malonyl-CoA. In human cells, acetyl-CoA is derived from glucose, which is broken down to pyruvate; pyruvate undergoes oxidative decarboxylation in the mitochondria to form acetyl-CoA. Since bacteria lack mitochondria, acetyl-CoA cannot be obtained this way. In bacterial cells, acetyl-CoA is instead produced from lactate, which is atypically broken down into acetate, CO2, and H2, as observed in Clostridium.

The created acetate is activated under the action of the enzyme *thiokinase*, in the presence of ATP, resulting in acetyl-CoA (necessary for the synthesis of fatty acids), AMP and pyrophosphate.

$$\text{Acetate} + \text{ATP} \xrightarrow{\textit{Thiokinase}} \text{Acetyl-CoA} + \text{AMP} + H_4P_2O_7$$

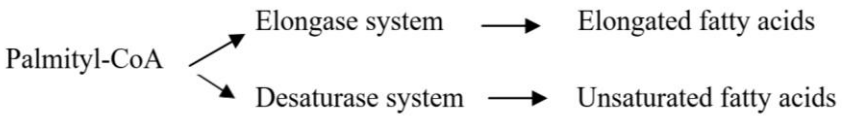
Malonyl-CoA is obtained in the same way as in human cells, i.e. by carboxylation of acetyl-CoA under the action of the enzyme *acetyl-CoA carboxylase*.

$$\text{Acetyl-CoA} + CO_2\ \text{ATP} \xrightarrow[\textit{Carboxylase}]{\textit{Acetyl-CoA}} \text{Malonyl-CoA} + \text{ADP} + \text{P}$$

Seven molecules of malonyl-CoA and one molecule of acetyl-CoA are required to synthesize one molecule of palmitic acid. The palmitic acid synthesis system (PASS) is responsible for this process.

$$7\ \text{Malonyl-CoA} + 1\ \text{Acetyl-CoA} \xrightarrow{\text{PASS}} \text{Palmitoyl-CoA} + \text{CoA-SH}$$

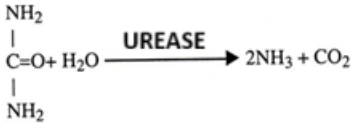
Fatty acids with a higher number of C-atoms or unsaturated fatty acids can be obtained from palmitoyl-CoA, depending on which system will be involved in the process (elongase system or systemic saturase).



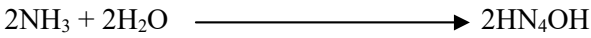
We have seen how the starting products for the synthesis of triglycerides are obtained: glycerol and fatty acids. After they have been obtained, the further synthesis of triglycerides in the bacterial cell takes place in the same way as in humans.

Urea catabolism

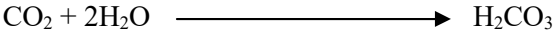
Urea is the end product of protein, specifically amino acid, breakdown in the human body. It enters saliva from the blood and is subsequently transported into dental plaque. Additionally, urea in the biofilm can be produced during metabolic processes such as arginine catabolism. The bacterial enzyme *urease* catalyzes the breakdown of urea into two molecules of ammonia (NH3) and one molecule of CO2.



The resulting product acts strongly alkaline because the two ammonia molecules in aqueous solution give the base ammonium hydroxide (NH_4OH).



The released CO2 does not significantly alter the alkaline environment, as only a small fraction reacts with water to form weak carbonic acid.

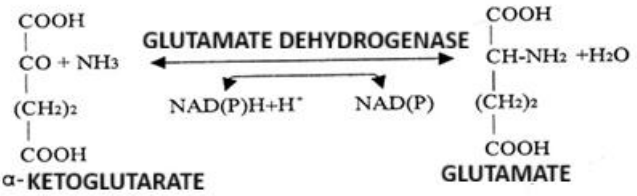


The breakdown of urea by bacteria in the biofilm leads to alkalization of the biofilm microenvironment. The released ammonia can also serve as a substrate for the synthesis of amino acids required by the bacteria.

Amino acids synthesis

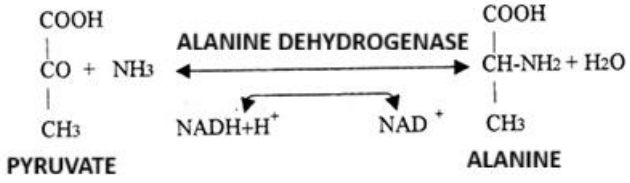
Bacteria in the biofilm possess enzymatic systems for amino acid synthesis, a metabolic feature typical of bacteria that utilize nitrogen compounds. For amino acid synthesis, adequate amounts of α-keto acids and ammonia are required. α-Keto acids are produced by bacteria that catabolize sugars and are released as unnecessary final metabolites into the biofilm microenvironment. These α-keto acids can then be taken up by other bacteria in the biofilm and used for amino acid synthesis. By incorporating ammonia into a keto acid, the corresponding amino acid is produced. A typical example of this process is the incorporation of ammonia into α-ketoglutaric acid, catalyzed by the bacterial enzyme *glutamate dehydrogenase*, yielding glutamate. This process is known as

reductive amination of ammonia.

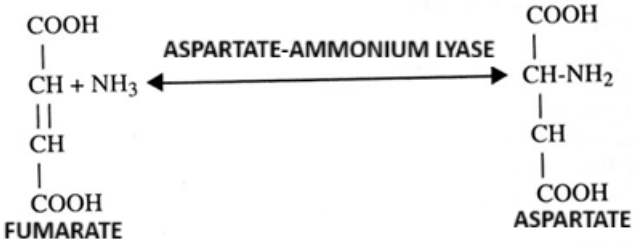


In this way, with the help of reductive ammoniation, a strong keto acid is removed and a new amino acid is obtained, necessary for further metabolism of individual microorganisms in the dental plaque.

The amino acid alanine is obtained by reductive amination of pyruvate. The enzyme *alanine dehydrogenase* catalyzes this reaction.



By amination of the fumaric ketoacid, the amino acid aspartate (aspartic acid) is synthesized under the action of the enzyme *aspartate-ammonium lyase*.

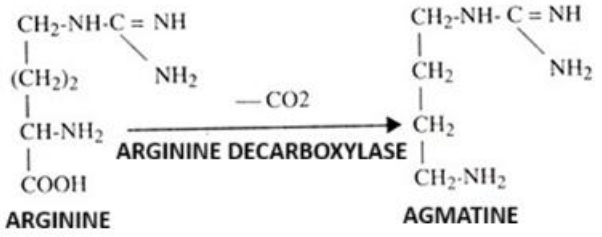


Synthesis of toxic amines

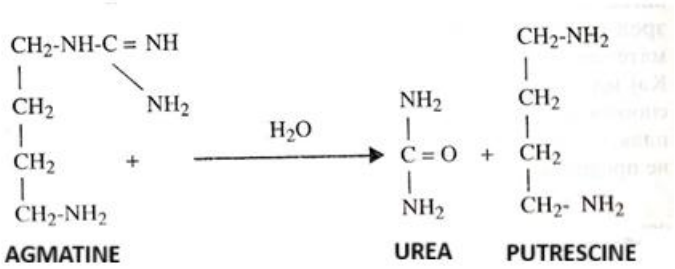
The synthesis of toxic amines is a metabolic process that occurs primarily in the biofilm, which contains bacteria capable of breaking down nitrogenous compounds, i.e., proteins. These bacteria possess specific enzymes, *proteases,* that degrade proteins into amino acids. The proteins targeted by bacterial enzymes

originate from saliva, gingival fluid, and residual food particles in the oral cavity. Protein degradation releases various amino acids, among which the basic amino acids ornithine, citrulline, arginine, and lysine are particularly important. These are diamino-monocarboxylic amino acids, containing an excess amino group relative to their carboxyl group. Their release into the biofilm causes alkalization, i.e., an increase in pH. Further catabolism of these basic amino acids through decarboxylation produces additional alkaline compounds in dental plaque. These compounds, known as toxic amines (including putrescine, cadaverine, and agmatine) lead to a more pronounced increase in the pH of the biofilm.

By decarboxylation of the amino acid arginine, the toxic amine agmatine is obtained.



Further breakdown of agmatine yields urea and toxic amine putrescine. Putrescine can also be obtained by decarboxylation of the basic amino acid citrulline. Cadaverine, on the other hand, is obtained by decarboxylation of the basic amino acid lysine.



Toxic amines have a harmful effect on the supporting apparatus of tooth.

CHANGES IN pH IN THE BIOFILM ENVIRONMENT

Changes in pH within the microenvironment of the biofilm significantly influence the development of the two most common oral diseases: periodontal disease and dental caries. A detailed analysis of electrochemical changes in dental plaque provides insight into the biochemical basis of the etiopathogenesis of these diseases. Therefore, the change in plaque pH can be understood through two approaches: the classic concept and the concept based on metabolic products in the biofilm.

Classic concept of pH change in biofilm

The classic concept, which explains the changes in the microenvironment of the biofilm, is based on the stages of biofilm formation and the type of bacteria that prevail therein. According to this concept, the value of the electrochemical reaction or pH in the biofilm depends on two factors:

1. Is the biofilm mature or immature?
2. Is catabolism of nitrogenous compounds or catabolism of carbohydrates predominant in the biofilm?

Immature biofilm

Immature biofilm is a thin, porous plaque on the teeth that forms shortly after brushing. It contains only a small number of bacteria and proteins and lacks extracellular polysaccharides such as dextran and levan. Consequently, microspaces exist between the bacteria, giving the biofilm a porous appearance. The diffusion capacity of this immature biofilm is highly pronounced.

When bacteria in the biofilm primarily metabolize carbohydrates, they produce various acidic metabolites, predominantly lactate and other keto acids. These potentially harmful acids readily diffuse from the biofilm microenvironment into saliva. Concurrently, salivary bicarbonates diffuse into the biofilm microenvironment, providing a buffering effect that can alkalize the local environment. Consequently, the pH of immature biofilm, despite acid production, remains largely similar to salivary pH. Should the biofilm pH decrease due to acid accumulation, saliva, through its flushing action and bicarbonate content, rapidly

neutralizes the acidic conditions. Thus, the biofilm pH is regulated. Experimental studies have demonstrated that following rinsing with a 10% glucose solution, biofilm pH decreases within 5–20 minutes due to bacterial glucose catabolism and lactate production. Subsequently, the reduced pH gradually rises over 30–60 minutes, driven by salivary bicarbonates that promote alkalization. The graph depicting these biofilm pH fluctuations is referred to as the Stephan curve (Figure 11.12). If salivary flow into the oral cavity is impaired, low biofilm pH values persist for a significantly longer duration.

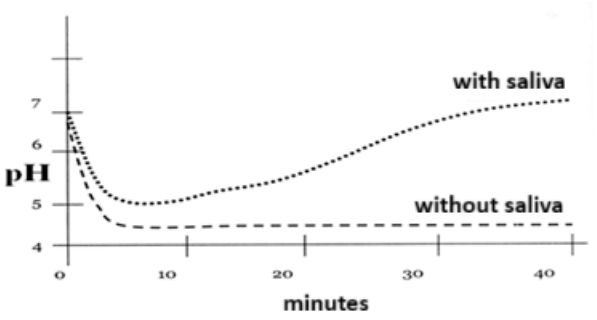


Figure 11.12: *pH changes in the biofilm after rinsing the teeth with 10% glucose solution with and without presence of saliva in the oral environment*

If nitrogen compound catabolism predominates in the biofilm, basic metabolites, including basic amino acids, ammonia, and toxic amines, are released. These metabolites do not accumulate in the immature porous biofilm but diffuse into the saliva, while salivary phosphate buffers diffuse into the biofilm. Consequently, biofilm pH does not rise significantly above salivary pH, and any increase that occurs is transient.

A mature biofilm

The mature biofilm develops from the immature biofilm and contains extracellular polysaccharides. These polysaccharides occupy the microspaces between bacteria, serve as a cementing matrix, and increase the biofilm volume. Consequently, the mature biofilm is non-porous, resulting in minimal diffusion capacity.

During carbohydrate catabolism, acidic metabolites, including lactate and other keto acids, accumulate as their diffusion into saliva is restricted, and salivary bicarbonate penetration is hindered. Due to this, there is an accumulation of acidic

metabolites in the mature biofilm. This leads to a substantial and prolonged decrease in biofilm pH, which can reach approximately 5.

When nitrogen compound catabolism predominates in the mature biofilm, basic metabolites, including basic amino acids, ammonia, and toxic amines, are released. Due to the limited diffusion capacity of the mature biofilm, these metabolites accumulate within its microenvironment, resulting in a marked increase in pH, which can reach up to 9. This elevated pH has a protective effect on tooth enamel but may be detrimental to the gingiva.

In the mature biofilm, pH fluctuations are more pronounced than in the immature biofilm, ranging from 5 to 9, depending on whether bacterial metabolites are predominantly acidic or basic. (Figure 11.13).

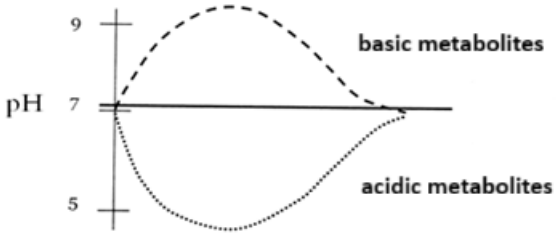


Figure 11.13: *Changes in pH in the mature biofilm depending on the metabolic activities of the bacteria*

A concept of pH change in biofilm based on the composition of its metabolic products

This concept does not contradict the classical explanation; rather, it refines and details the impact of specific metabolic products in the dental biofilm on its electrochemical changes. According to this approach, **pH** fluctuations in the biofilm are explained by the types of metabolic products present.

When analyzing biofilm pH, two terms are used based on the time since the last intake of carbohydrate-rich food. "Saturated plaque" refers to plaque on teeth 2–2.5 hours after consuming carbohydrates, whereas "unsaturated plaque" refers to plaque present 8–12 hours after such intake. The pH of saturated plaque ranges from 6 to 7, while unsaturated plaque has a pH of 7 to 8.

Saturated plaque contains a relatively high concentration of acetate compared to lactate. The predominant amino acids in the plaque are glutamic acid

and proline. Ammonia is also present at a notable concentration. The higher level of acetate results from the metabolic end products of both amino acids and carbohydrates. These end products accumulate in the plaque at concentrations greater than in saliva and are continuously generated during the metabolism of intracellular and extracellular bacterial carbohydrates, as well as salivary glycoproteins.

A decrease in plaque pH

There are two significant factors that affect the reduction of plaque pH:

1. Presence of exogenous fast-fermenting carbohydrates, especially sucrose
2. The low buffering capacity of unstimulated saliva

A decrease in the pH of the biofilm is primarily associated with increased production of **lactic acid (lactate).** Concurrently, as lactate concentration rises in the plaque, the levels of acetate and propionate diffusing from the plaque into the saliva decrease. These processes influence the pH changes within the central region of the plaque because:

1. Acids that have higher pK values have better buffering capacity
2. Acetate and propionate have a much higher pK than lactate (that is, they have a better buffering capacity)
3. The amount of lactate increases, which has a low pK value (that is, it has a weaker buffering capacity).

Acids with higher pK values exhibit greater buffering capacity, as they can absorb hydrogen ions released by the dissociation of acids with lower pK values. Consequently, the diffusion of acetate (which has superior buffering capacity) from dental plaque into saliva further contributes to the pH decrease in the central region of the plaque. As the plaque pH declines, the concentrations of amino acids and ammonia also decrease, partly due to their utilization in bacterial anabolic processes.

Increase in pH values of the plaque

The previously mentioned factors also contribute to the increase of pH in the biofilm. Ammonia, a highly alkaline compound, can neutralize acids within the biofilm. It is produced through the hydrolysis of urea and the deamination of amino acids in the plaque. Other basic compounds in the biofilm include various amines, generated by

decarboxylation of amino acids.

Glutamic acid, the predominant amino acid in the plaque, plays a key role as an amino donor in the synthesis of multiple amino acids from other organic acids (keto acids) within the plaque. This process helps neutralize strong organic acids, leading to an increase in plaque pH. Delta-aminovaleric acid, derived from proline, is also important for elevating plaque pH, as its formation involves the breakdown of lactate (Figure 11.14).

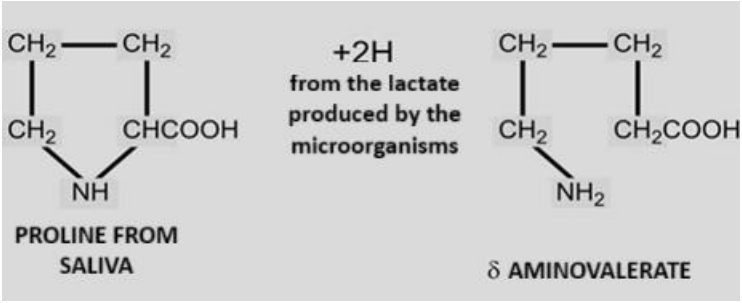


Figure 11.14: *Formation of delta-aminovaleric acid through the reaction of proline from saliva with hydrogen ions released during lactate degradation*

An increase in pH may also be due to the presence of the bacterium Veillonela, which further metabolizes lactate to acetate and butyrate, acids weaker than lactate.

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12 BIOFILM AND ORAL HOMEOSTASIS

SALIVARY CLEARANCE AND ORAL HOMEOSTASIS

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SALIVARY CLEARANCE AND ORAL HOMEOSTASIS

Understanding the variability in salivary clearance of dietary carbohydrates, acidic metabolic by-products of plaque, and therapeutic agents (e.g., fluorides) enables the explanation of individual differences in susceptibility to oral diseases, as well as the differential involvement of various oral surfaces.

Numerous substances enter the oral cavity daily. Some, such as sucrose and acidic metabolites, can be detrimental to oral tissues, whereas others, such as fluorides, exert protective effects. Many of these substances dissolve in saliva, allowing them to diffuse into or interact with oral tissues. Freshly secreted saliva, together with the act of swallowing, reduces the concentration of exogenous substances in the oral cavity. This process is referred to as salivary clearance. In Macedonian, several synonymous expressions are used to describe this phenomenon, including: altered concentration of a substance in saliva, self-cleaning, or removal of specific dissolved particles from the oral cavity via saliva.

The rapid salivary clearance, i.e. cleaning of harmful substances, is suitable for oral health. But rapid clearance is not suitable when it comes to protective substances, because they are thus eliminated from the oral cavity in a short time.

Salivary clearance models

A. Swenander-Lanke Model

This was the first simple model for salivary clearance, described in the mid-20th century. According to the model, dissolved sucrose in saliva (volume V) establishes an initial concentration of sucrose in saliva (C_0). The rate of saliva secretion has a constant (F), representing the volume of saliva removed from the oral cavity through swallowing. The sucrose concentration (Ct) at a given time after saliva secretion decreases and can be expressed by the formula:

$$Ct = C_0 \cdot e^{-Ft/V}$$

Experimental studies show that plotting the logarithm of sucrose concentration against time produces a straight line. This linearity indicates that beyond a certain point, the sucrose concentration in saliva stabilizes and no longer decreases. The straight line begins once saliva secretion returns to an unstimulated rate, as the previously administered sucrose solution initially stimulated secretion.

B. Dawes Model

A more recent model describes the swallowing process as being equivalent to the action of an incomplete siphon (Figure 5.1). After a swallow, the mouth retains a minimum volume of saliva, called the residual volume (Resid). Saliva then flows into the mouth at a rate dependent initially on the stimulating effect of the ingested substance but later, once the concentration is below the taste threshold, or after taste adaptation has occurred, on the unstimulated flow rate. The volume of saliva in the mouth thus increases until a maximum volume (Vmax) is reached. This stimulates the subject to swallow, which clears some of the substance from the oral cavity. The remainder (dissolved in the residual volume of saliva) is then progressively diluted by more saliva entering the mouth until Vmax is reached again, and another swallow occurs. The Dawes model has been used to describe with considerable accuracy the clearance of substances, including sucrose, which do not bind to oral surfaces. Other studies have indicated that with some substances, clearance may occur in two stages, rapidly from the bulk of the saliva, and more slowly from stagnation areas.

Swallowing

Swallowing is a complex neuromuscular process that not only transports food from the oral cavity to the digestive tract but also protects the respiratory tract from aspiration of food, liquids, and saliva.

In the mouth, following mastication, the preparatory phase of swallowing occurs, involving the formation of a saliva-moistened food bolus and its positioning on the dorsum of the tongue. The oral phase of swallowing then proceeds with elevation of the tongue and propulsion of the bolus into the pharynx. The pharyngeal phase is largely regulated by the swallowing center in the pons.

Swallowing of saliva, in the absence of ingested food or drink, is likely triggered by stimulation of laryngeal receptors by saliva present on the posterior third of the tongue dorsum.

Dysphagia, or difficult swallowing, can occur in the elderly due to a decrease in fine motor control or due to some diseases, such as Parkinson's disease, multiple sclerosis, stroke or trauma.

Elimination of Substances Capable of Binding to Oral Structures

Fluorides

For the clearance of fluorides in saliva, *Dawes Model* requires refinement, as fluoride concentrations remain elevated for several hours after rinsing with fluoride-containing liquids or pastes, forming a salivary reservoir. During the initial clearance phase, when salivary fluoride levels are high, fluoride diffuses into dental plaque, adheres to the oral mucosa (from which it can later re-enter saliva), or forms calcium fluoride deposits on the teeth. Consequently, fluoride clearance is delayed, both due to these deposits and the gradual release of fluoride from the oral mucosa and calcium fluoride, which sustains elevated salivary fluoride concentrations. The majority of ingested fluoride is absorbed in the digestive tract, with only a small fraction (<0.2%) returning to saliva via ultrafiltration through the acinar cells of the salivary glands.

Chlorhexidine

Chlorhexidine, applied as a solution, gel, or varnish, effectively reduces plaque formation, thereby contributing to the prevention of caries and periodontal disease. Unlike other antimicrobial agents, chlorhexidine exhibits strong adherence to oral surfaces, including both teeth and the epithelial cells of the oral mucosa. This binding prolongs its presence in the oral cavity, resulting in sustained effects and a slower clearance rate.

Microorganisms and epithelial cells

Saliva secreted by the glands is initially sterile; however, mixed whole saliva contains approximately 10^9 bacteria per milliliter. For bacterial survival in the oral cavity, adherence to oral surfaces is essential during unstimulated salivary flow. Oral epithelial cells are continuously desquamated, with cells from the most superficial layer persisting for only about three hours. Each desquamated epithelial cell entering the saliva carries roughly one hundred attached bacteria. Consequently, saliva contains approximately three times more bacteria associated with epithelial cells than free-floating bacteria. The majority of salivary bacteria originate from epithelial cells rather than tooth surfaces. Following prophylactic procedures, if oral hygiene is not maintained, plaque accumulation on the teeth

will increase until a balance is established between bacteria migrating from dental plaque into saliva and bacteria proliferating within the plaque. Such salivary clearance facilitates the removal of bacteria and desquamated epithelial cells from the oral cavity. Consequently, salivary clearance is crucial for maintaining oral microbial balance, and individuals with hyposalivation exhibit increased levels of bacteria and epithelial cells in their saliva. Salivary secretion is markedly reduced during sleep, leading to an accumulation of bacteria and desquamated epithelial cells overnight. Morning halitosis arises primarily because epithelial cells provide a substrate for gram-negative bacteria, which produce volatile sulfur compounds responsible for the unpleasant odor.

Factors affecting salivary clearance

The most important factors affecting salivary clearance are: the residual and maximum volume of saliva, the secretion of unstimulated and stimulated saliva, as well as the degree of bonding with oral surfaces of the substance being cleaned.

Residual saliva volume-saliva remaining in the oral cavity after swallowing

In a study involving 40 healthy subjects, the residual volume of saliva was measured. The mean value was 0.8 ml, with considerable interindividual variability ranging from 0.4 to 1.4 ml. According to the *Dawes model*, these variations also influence the half-time of clearance that is, the time required for the concentration of a substance, such as salivary sucrose to decrease by half in the oral cavity (Graph 12.1). It was observed that individuals with faster swallowing rates and smaller residual volumes clear the substance from the mouth more rapidly.

Maximum saliva volume-saliva volume present immediately before swallowing (Vmax)

Another important factor influencing salivary clearance is the volume of saliva present in the mouth immediately prior to the initiation of swallowing. The

mean value of this pre-swallow volume is 1.1 ml, with a range of 0.5–2.1 ml, reflecting interindividual variability similar to that of the residual volume. It was found that individuals with a higher residual volume also tend to have a higher maximum saliva volume. Conversely, those with a lower maximum volume before swallowing clear residual particles from the oral cavity more rapidly (Figure 12.1).

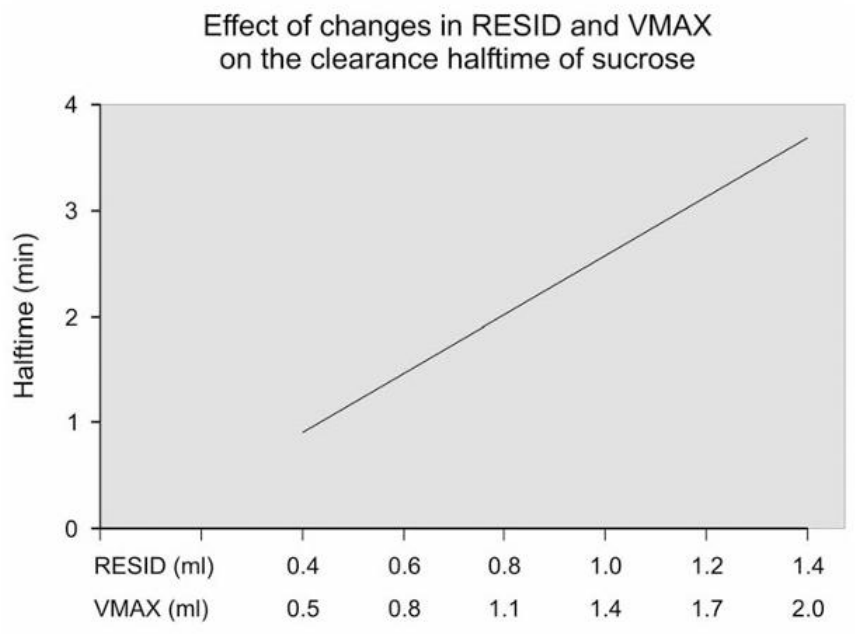


Chart 12.1: *Computer simulations were conducted to evaluate the impact of variations in the residual post-swallowing volume (Resid) and the maximal pre-swallowing volume (Vmax) on the self-clearance half-time of sucrose following a 10% sucrose mouthwash. The self-clearance half-time represents the duration required for the concentration of the substance (in this case, sucrose) to decrease by half.*

Unstimulated saliva secretion

The volume of unstimulated saliva secreted ranges from 0.3–0.4 ml/min and exhibits interindividual variability. The quantity of saliva secreted influences both the act and frequency of swallowing. According to *Dawes model*, a reduced rate of unstimulated salivary secretion results in a prolonged half-life for sucrose clearance (Figure 12.2). In individuals with marked hyposalivation, where

unstimulated saliva secretion falls below the minimum threshold of 0.05 ml/min, carbohydrate clearance is significantly delayed, rendering these individuals more susceptible to caries.

It should be emphasized that even when two individuals have the same rate of unstimulated salivary secretion, differences in salivary clearance half-life may occur depending on the levels of residual and maximal saliva volumes; lower volumes correspond to faster clearance, while higher volumes result in slower clearance.

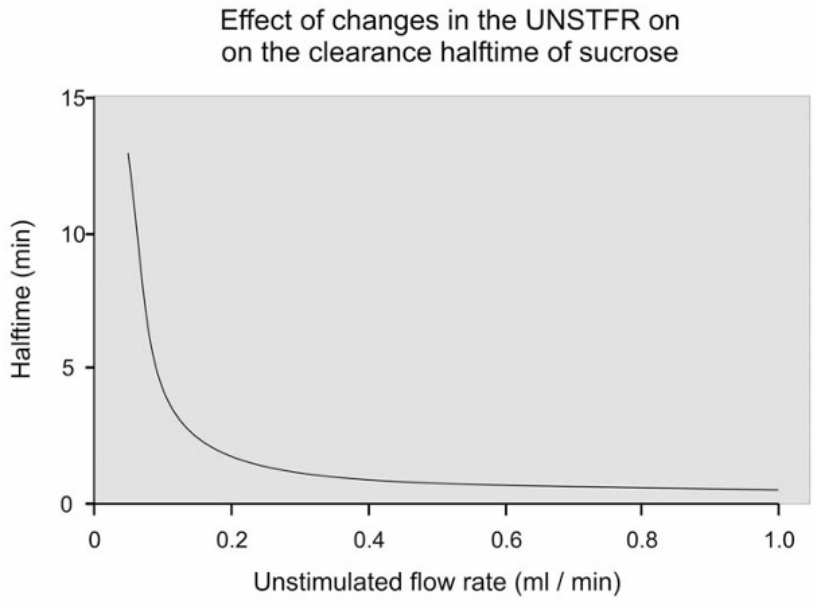


Chart 12.2: *Computer simulation of the effect of changes in unstimulated salivary secretion on sucrose clearance following a 10% sucrose mouthwash. The simulation determined average values for secreted unstimulated saliva (0.32 ml/min.), Resid (0.8 ml), and Vmax (1,1 ml). The curve shows that the smaller the salivary flow rate, the more the cleaning, that is, the clearance of the substance (in this case sucrose) is delayed.*

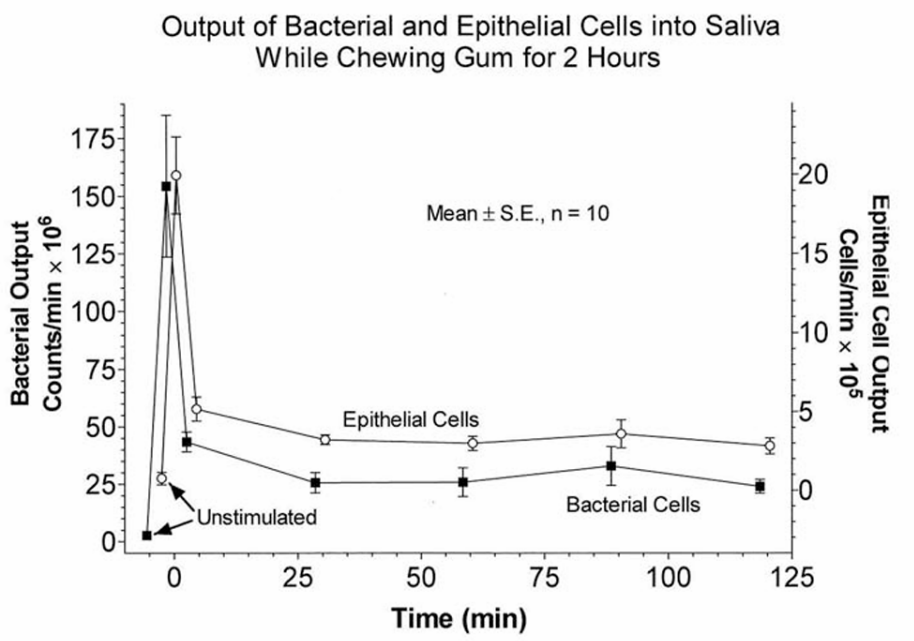
A large number of salivary antimicrobial peptides, at normal values of residual and maximum saliva volume and physiological values of secreted unstimulated saliva, have a low half-time of salivary clearance (2.2 minutes).

Stimulated saliva secretion

Lagerlöf and colleagues demonstrated through computer modeling that stimulated salivary secretion significantly influences the clearance of fluorides from the oral cavity. Rapid salivary clearance of sucrose and fluoride, resulting from stimulated salivation, limits the diffusion of these substances in saliva. Consequently, fluoride tablets intended for local oral use should avoid ingredients that strongly activate taste receptors, as this would trigger additional salivary secretion. Such tablets should dissolve gradually in saliva and preferably should not be chewed.

Studies examining bacterial content in saliva during stimulation by chewing paraffin balls showed that the number of bacteria and desquamated epithelial cells rises sharply during the initial minutes of chewing and then declines (Graph 12.3). This underscores the importance of carefully controlling saliva secretion when collecting samples for biochemical analysis.

The act of chewing gum initially stimulates salivary secretion up to 12 times higher than the rate of unstimulated saliva. During extended chewing, saliva output remains elevated at approximately 2–3 times the unstimulated level. Interestingly, chewing gum does not substantially enhance the mixing of secretions from different salivary glands; however, the overall increased saliva volume effectively accelerates salivary clearance.



Graph 12.3 *– The amount of bacteria and epithelial cells in saliva after chewing gum stimulation for 2 hours.*

Salivary film

For many years, oral biologists assumed that dental plaque is uniformly covered by saliva, with a constant composition and only variations in the quantity of secreted saliva. However, it has been demonstrated that plaque is almost continuously coated by a very thin salivary film, whose composition changes locally as specific substances diffuse into or out of the plaque. Considering an average saliva volume of approximately 1 ml and an adult oral surface area of 200 cm^2, the resulting thickness of the salivary film covering oral surfaces is about 0.1 mm.

Studies have shown that the thickness of the salivary film varies across different oral surfaces, ranging from 70 μm on the posterior third of the tongue's dorsum to 10 μm on the mucosa of the anterior hard palate. In patients reporting severe dry mouth (xerostomia), the average residual saliva volume is about 71% of the normal level, indicating that dryness affects specific oral surfaces, such as the anterior hard palate and the anterior third of the tongue, rather than the entire

mouth. During unstimulated salivary secretion, the thickness of the salivary film changes rapidly (0.8–8.0 mm/min) across different oral surfaces. Very low salivary flow rates significantly impact the clearance of ingested carbohydrates, locally applied fluorides, and particularly the acidic metabolic byproducts released from plaque.

Clearance of Substances from Oral Surfaces

Carbohydrate intake through diet

Caries develops due to the demineralizing action of organic acids produced in the dental biofilm during bacterial breakdown of carbohydrates, particularly sucrose. On oral surfaces with rapid salivary clearance, only a small amount of ingested sucrose penetrates the dental plaque, reducing the potential for acid formation. Research has shown that sucrose, when introduced in different forms, is unevenly distributed throughout the oral cavity and cleared at different rates from various surfaces. Salivary clearance occurs more quickly on the lingual surfaces of teeth than on the buccal surfaces, with the exception of the buccal surfaces of the upper molars, where parotid saliva is secreted. Apart from these upper molar surfaces, the buccal surfaces are predominantly covered by highly viscous saliva from the minor mucous glands, whereas the lingual surfaces are mainly bathed in secretions from the two major salivary glands: the submandibular and sublingual glands.

Figure 12.1 presents the mean sucrose clearance values in total saliva and on six specific oral surfaces after rinsing with 10% p/p sucrose in ten individuals. Areas with faster salivary flow show lower initial sucrose concentrations and quicker clearance. For comparison, the lingual surfaces of the lower incisors and the buccal (vestibular) surfaces of the lower molars are highlighted. On average, the salivary film changes at a rate of 8 mm/min on the lingual surface of the front teeth, whereas on the vestibular surfaces of the molars, the rate is only 1 mm/min.

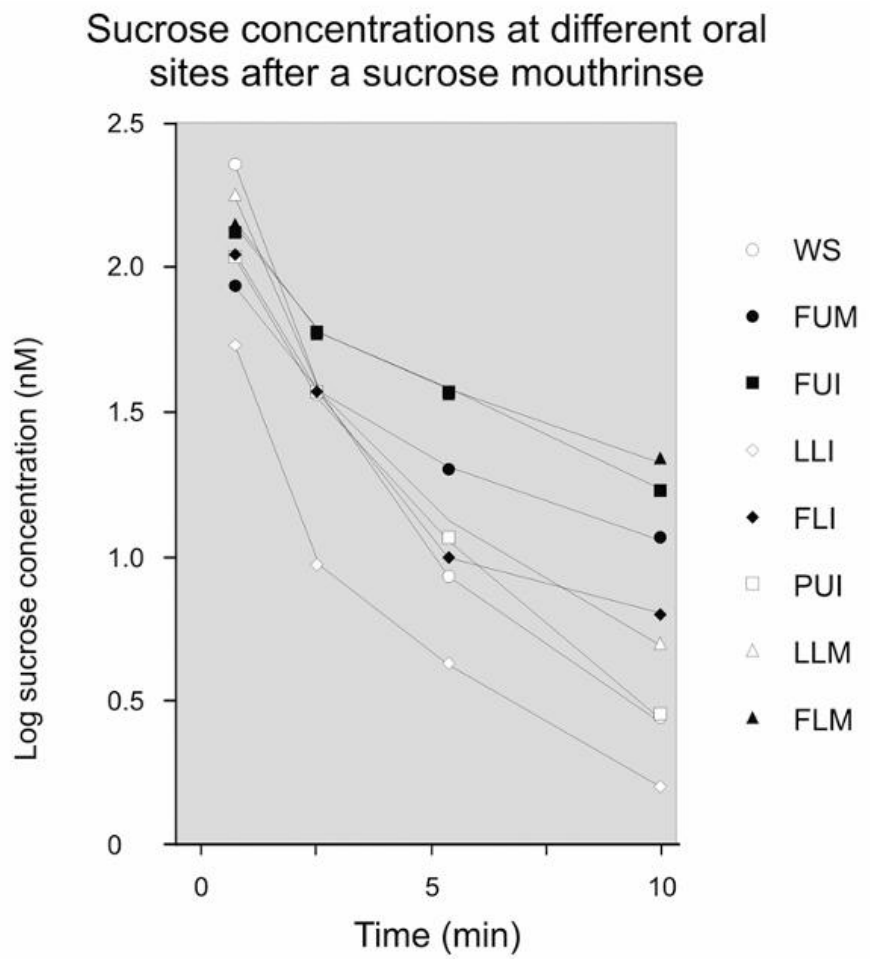


Figure 12.1: *Sucrose concentrations in saliva on different surfaces after rinsing with 10% sucrose solution*. WS = whole saliva; FUM = facial upper molars; FUI = facial upper incisors; LLI = lingual lower incisors; FLI = facial lower incisors; PUI = palatal upper incisors; LLM = lingual lower molars; FLM = facial lower molar

HARMFUL EFFECTS OF BIOFILM ON PERIODONTAL TISSUES

The high metabolic activity within the biofilm microenvironment leads to the release of numerous harmful metabolites that negatively impact tooth enamel, causing demineralization. In addition, these metabolites can damage other oral structures, particularly the gingiva, periodontal fibers, cementum, and alveolar bone. Bacterial enzymes and toxins contribute to the breakdown of these tissues, potentially resulting in periodontal disease. Since biofilm bacteria harm both the tooth enamel and its supporting structures, it can be concluded that the biofilm disrupts oral homeostasis.

Diseases affecting the tooth-supporting structures are among the most common in humans, with bacteria from the biofilm being the primary cause. Among the harmful substances released from the plaque, hydrolytic enzymes are particularly significant. These enzymes degrade the structural biomolecules that form both soft and hard oral tissues. Notably, hyaluronidase and chondroitin-sulfatase contribute to the breakdown of hyaluronic acid and chondroitin sulfate, which are essential structural components of oral tissues. Bacterial enzymes such as collagenase and elastase exert especially detrimental effects on the tooth-supporting apparatus by degrading the structural proteins collagen and elastin.

Apart from hydrolytic enzymes, the bacteria in the biofilm also release other harmful substances such as endotoxins or lipopolysaccharides (Figure 12.2). Endotoxins are components of the outer membrane of some bacteria in the biofilm. Endotoxin consists of three components:

- O-polysaccharide chain
- outer and inner polysaccharide core
- hydrophobic lipid A.

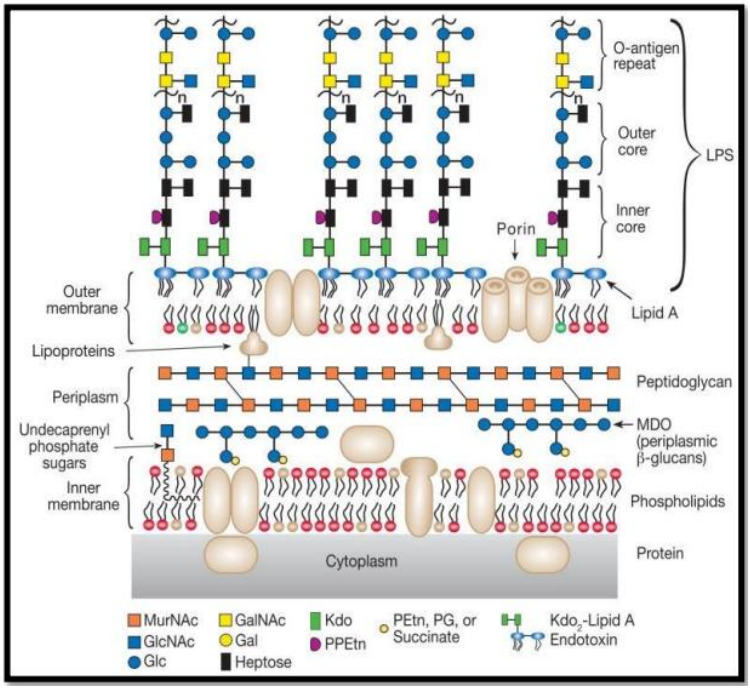


Figure 12.2: *Schematic representation of the surface structures of gram negative bacteria (endotoxin)*

Hydrophobic lipid A constitutes the toxic component of the endotoxin molecule, which is also referred to as modulin. This endotoxin stimulates leukocytes to release pro-inflammatory mediators, known as cytokines. The key cytokines involved are interleukin 1, prostaglandin E2, and tumor necrosis factor (TNF). These mediators are considered the primary factors responsible for the destructive changes in periodontal tissues, particularly the resorption of alveolar bone.

Additionally, the enzyme matrix metalloproteinase (MMP) plays a role in degrading proteins of the periodontal tissues, including collagen, elastin, and gelatin. MMP is initially secreted in an inactive form and cannot degrade extracellular periodontal proteins until it is activated. Activation requires partial proteolysis, which involves the cleavage of a segment of its polypeptide chain. This activation process is mediated by a chymotrypsin-like enzyme produced by bacteria within the biofilm.

The end products of the metabolism of proteolytic bacteria, ammonia and toxic amines have a harmful effect on the periodontium, especially on the gingiva.

HARMFUL EFFECTS OF BIOFILM ON DENTAL HARD SUBSTANCES

Calcium salts in dental plaque and their role in demineralization and remineralization

The high metabolic activity within dental plaque induces changes in tooth enamel. Beyond enamel, these metabolic processes also affect the gingiva, and in individuals with periodontal disease, they extend to the alveolar bone.

Because of its location and direct exposure to plaque-derived products, enamel is the first tissue to be affected by the harmful effects of these metabolic byproducts.

Enamel is primarily composed of inorganic material, with calcium being the main constituent. The acidic metabolic products of dental plaque—such as lactate, acetate, propionate, and butyrate—cause a local decrease in pH, creating an acidic environment. When the pH drops below 5.5, enamel is adversely affected as the calcium salts, specifically hydroxyapatite crystals, begin to dissolve. This process can initiate dental caries.

Calcium phosphate, the principal compound in the inorganic portion of enamel as well as in other hard tissues of the body, exists in multiple forms. Its properties depend directly on the calcium-to-phosphorus (Ca/P) ratio, which is summarized in Table 12.1.

Table 12.1: Ratio of calcium to phosphorus in individual calcium phosphate salts

Name	Formula	Ca/P quotient
Phosphoric acid	H_3PO_4	0
Monocalcium phosphate	$Ca(H_2PO_4)_2$	0.50
Dicalcium phosphate	$CaHPO_4$	1.00
Octacalcium phosphate	$Ca_8H_2(PO_4)_6 \cdot H_20$	1.33
Tricalcium phosphate	$Ca_3(PO_4)_2$	1.50
Hydroxyapatite	$Ca_{10}(PO_4)_6(OH)_2$	1.66
Alkaline calcium phosphate	$(Ca_2PO_4OH) \cdot H_20$	2.00

The properties of calcium salts depend not only on the calcium-to-phosphorus ratio but also on the pH of the surrounding environment. When any calcium phosphate salt is exposed to an acidic environment, it dissolves, forming a different type of salt with a lower calcium and phosphate content. This occurs as calcium ions are released into the surrounding medium, which increases the solubility of the resulting salt.

Conversely, when a calcium phosphate salt is exposed to a basic environment, a new form of the salt with reduced solubility is produced. In this case, the Ca/P ratio of the resulting salt increases because the base promotes the release and loss of phosphate ions.

It is evident that the pH of the dental plaque directly influences the composition and properties of the corresponding calcium salts, including their fundamental characteristics. In dentistry, the behavior of hydroxyapatite, the primary mineral component of hard tissues, is particularly significant.

In bone tissue, the metabolic activity of osteoblasts is tightly regulated, and the pH is maintained within a narrow range of approximately 6.5 to 7.5. In contrast, the metabolic activity within dental plaque is much less controlled, leading to wide fluctuations in pH, typically ranging from 5 to 9, and occasionally beyond.

A decrease in the local plaque pH inevitably affects hydroxyapatite. Exposure to acidic metabolic products causes hydroxyapatite to lose calcium ions, resulting in the formation of dicalcium phosphate—a salt with a markedly altered and less favorable calcium-to-phosphorus ratio:

$$Ca_{10}(PO_4)_6(OH)_2 + 8H \rightarrow 6\ CaHPO_4 + 2H_2O + 4Ca$$

The dicalcium phosphate formed is stable and largely insoluble under neutral pH conditions. However, in environments with even mild acidity, this salt tends to dissociate continuously, leading to a progressive release of calcium ions and, ultimately, the loss of the entire mineral content:

$$CaHPO_4 + H \rightarrow Ca + H_2PO_4$$

The initial breakdown of hydroxyapatite and its transformation into a dicalcium phosphate salt can temporarily have a protective effect. It is about $CaHPO_4$, a salt with poor solubility in a neutral environment, which covers the hydroxyapatite in the deeper layers. However, this is only a temporary effect of dicalcium phosphate, because its protection is manifested only in conditions of complete removal of the dental plaque. Reformation of plaque, even in small amounts, will quickly indicate that the site of dicalcium phosphate formation is a site of lower resistance.

This is confirmed by the fact that the immature plaque, which does not contain extracellular polysaccharides and is characterized by a high diffusion capacity for plaque metabolites as well as for salivary bicarbonates, can lead to the dissociation of $CaHPO_4$ and cause a progressive loss of this protective covering. With the complete loss of $CaHPO_4$, the hydroxyapatite in the deeper layers becomes exposed to new damage.

However, under certain conditions, alkaline metabolites are also produced within dental plaque. When exposed to these basic metabolites, hydroxyapatite undergoes partial transformation into dicalcium phosphate, thereby creating conditions favorable for the reformation of hydroxyapatite. In this process, a portion of phosphate ions is released and lost, as illustrated in the following reaction:

$$10CaHPO_4 + 8OH \rightarrow Ca_{10}(PO_4)_6(OH)_2 + 4HPO_4 + 6H_2O$$

Since the hydroxyapatite molecule consists of 18 ions—10Ca, 6PO_4, and 2OH—all of these ions must be simultaneously present in the correct ratio at the same site for crystallization to occur. For this reason, the likelihood of hydroxyapatite formation being reinitiated on a significant scale is very limited. This is particularly unlikely within the complex and highly unfavorable microenvironment of dental plaque.

In contrast, during the growth and development of enamel, dentin, cementum, and normal bone tissue, crystallization proceeds under stable and controlled conditions that prevail in their respective environments. Such optimal conditions, however, are absent both in the oral cavity and in the microenvironment of dental plaque, making large-scale recrystallization improbable—especially not to the extent of the original transformation of hydroxyapatite into dicalcium phosphate.

During the formation of dicalcium phosphate, calcium ions diffuse into the plaque fluid and subsequently into the saliva. Consequently, in addition to the 6 CaHPO4 formed, four further units are required to provide sufficient material for the reconstruction of one hydroxyapatite molecule, Ca10(PO4)6(OH)2. Thus, the breakdown of two hydroxyapatite units is necessary to yield a total of 10 CaHPO4 and enable the re-formation of a single hydroxyapatite structure. However, this process can occur only under alkaline conditions. When the calculations of the preceding reactions are considered, it becomes evident that enamel recrystallization is inherently deficient, since it inevitably results in a net loss of mineral content and, consequently, progressive enamel degradation.

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Such alterations lead to the formation of cavitations, i.e., defects of varying size on the tooth surface. Clinically, these present as white spots, a phenomenon frequently encountered in dental practice. These white spots are visible solely as areas of altered coloration and do not suggest the presence of an enamel defect; consequently, during routine examination, the tooth may be erroneously considered healthy. The development of these spots is attributable to the prolonged action of metabolites from dental plaque on the enamel. Initially, there is a loss of calcium, followed by phosphate, which results in the formation of a new calcium-phosphate salt characterized by increased solubility.

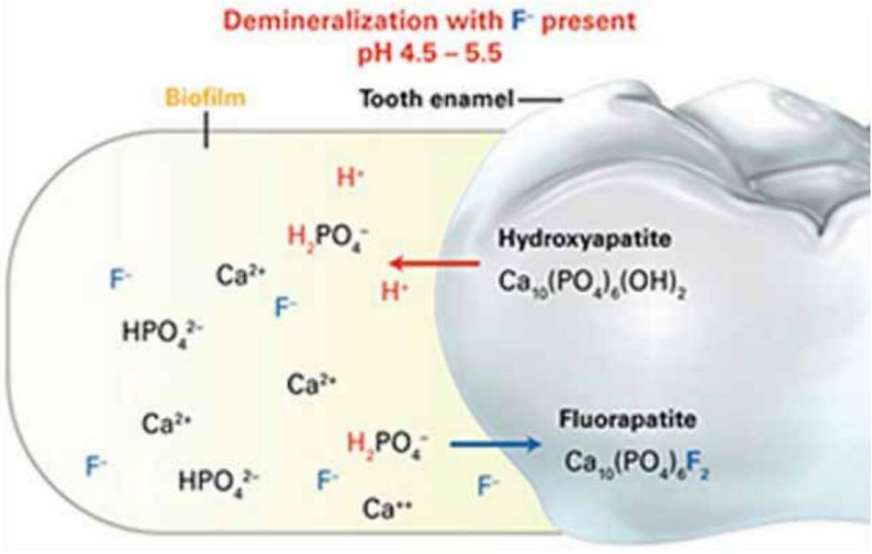

Figure 12.3: *Demineralization of hydroxyapatite at low pH values, in the presence of fluorides*

These white spots differ significantly in composition and properties from healthy enamel, as they predominantly consist of dicalcium phosphate. Given the characteristics of dicalcium phosphate, it follows that the formation of an initial plaque on such a white spot will rapidly lead to the loss of its surface layer. This process progresses to the development of a defect, which, in the presence of plaque bacteria, establishes conditions conducive to the initiation of a carious lesion.

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PREVENTION MEASURES AGAINST THE HARMFUL EFFECTS OF BIOFILM

Dental plaque develops as a result of bacterial colonization of the dental pellicle, in conjunction with the intake of dietary sugars, particularly sucrose. Oral hygiene also plays a crucial role, and it is often suboptimal.

The bacteria comprising the oral flora are present in the oral cavity even in the healthiest individuals. Colonization begins immediately after birth, forming the saprophytic flora thus establishing a biological equilibrium with the the host. This balance is maintained through the action of saliva and its immunologically active components, including immunoglobulins, complement, and lysozyme.

Diet, particularly one high in sucrose, represents a critical factor in dental plaque formation. In the absence of sucrose—a disaccharide by biochemical definition—the initial or "young" plaque cannot progress into a "mature" plaque. This process is explained by the fact that the hydrolysis of the glucosidic bond in sucrose releases the energy required for the polymerization of glucose into dextran and fructose into levan.

Specifically, bacterial enzymes such as dextran-sucrase and levan-sucrase catalyze the hydrolytic breakdown of sucrose into its constituent monosaccharides, glucose and fructose. Subsequently, the energy released during this reaction facilitates their polymerization into dextran and levan:

$$\text{Sucrose} \xrightarrow[\text{Levan sucrase}]{\text{Dextran Sucrase}} \text{Dextran} + n\ \text{Fructose}$$

$$\text{Sucrose} \longrightarrow \text{Levan} + n\ \text{Glucose}$$

These two enzymatic reactions may also be considered collectively, which is reasonable given that their initiation, progression, and formation of the respective products—dextran and levan—occur simultaneously. Accordingly, this reaction can be represented as follows:

$$\text{Sucrose} \xrightarrow[\text{Levan Sucrose}]{\text{Dextran Sucrose}} \text{Dextran} + \text{Levan}$$

Since this reaction also requires energy, which is released by breaking the glucosidic bonds from sucrose, thermodynamically, this reaction can be presented in the following way:

$$\text{Sucrose} + 7\ \text{kcal} \xrightarrow[\text{Levan Sucrose}]{\text{Dextran Sucrose}} \text{Dextran} + \text{Levan} - 1\ \text{kcal}$$

During the hydrolysis of sucrose, 7 kcal of energy is released, corresponding to the energy contained within this disaccharide. The synthesis of dextran consumes 4 kcal, while levan synthesis requires 2 kcal. Consequently, 1 kcal of energy remains, which serves to accelerate and direct the reaction toward the formation of dextran and levan.

It is evident that the combination of these critical factors—oral bacteria and diet—together with inadequate oral hygiene, inevitably leads to the formation of mature, gelatinous dental plaques. This raises the important question: "How can oral tissues be protected from the detrimental effects of mature dental plaque?"

1. Avoiding great amount of sugar components in the diet.
2. Adequate oral hygiene. Regular and correct mechanical removal of the initial plaque prevents maturing and formation of plaque whose products have a harmful effect on the teeth and periodontal tissues.
3. Application of fluorides. It is a preventive-therapeutic measure that can be implemented in two ways:
   - systemic application - fluoridation of drinking water, table salt, milk and use of fluoride tablets;
   - local application of fluoride solutions with different concentrations.

Systemic administration involves the regular introduction of fluorides into the body, which, following absorption in the digestive tract, reach the sites of tooth development. This process enables heteroionic substitution during odontogenesis, resulting in the formation of fluorapatite crystals, which exhibit increased hardness and reduced solubility compared to hydroxyapatite.

In contrast, local application targets the protection of the most vulnerable tooth enamel surfaces, thereby mitigating the deleterious effects of dental plaque.

It is important to emphasize that both systemic and local fluoride administration ensure the presence of fluoride ions in saliva as well as within dental plaque.

Fluoride metabolism

The administration of fluoride represents an effective strategy for caries prevention, with applications both systemically and locally.

Following the intake of fluoride via drinking water or fluoride-containing tablets, absorption occurs primarily in the small intestine, presumably through active transport. Highly soluble fluoride compounds, such as sodium fluoride, are rapidly and completely absorbed, whereas the absorption of calcium, magnesium, and aluminum fluoride is comparatively less efficient. In the acidic environment of the stomach, less soluble fluoride compounds dissociate, releasing fluoride ions that bind to hydrogen ions to form hydrogen fluoride. This compound readily penetrates cell membranes, allowing for the absorption of a larger proportion of ingested fluoride (approximately 60–75%). Dietary calcium, magnesium, and aluminum reduce fluoride absorption, whereas the presence of iron and phosphate enhances its uptake in the digestive tract.

After absorption from the digestive tract, fluoride enters the bloodstream and is distributed throughout the body. Its concentration in non-mineralized tissues and body fluids remains low. In plasma, fluoride exists in two forms: ionic (approximately 50%) and protein-bound (approximately 50%). Only the ionic form is biologically active. The total plasma fluoride concentration (including both ionic and protein-bound forms) ranges from 0.7 to 2.4 μmol/L, depending on the amount of fluoride ingested.

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Fluoride from the blood reaches the saliva, where its concentration is approximately 30–35% lower than in plasma. It also diffuses into the dental biofilm, where it exists in two forms: ionic (about 5%) and bound fluoride. Due to its high reactivity, fluoride binds to various biomolecules within the biofilm, including proteins, and can penetrate the cytoplasm of biofilm bacteria. In the plaque fluid, fluoride is present as calcium fluoride, and its concentration increases following the local application of concentrated fluoride solutions to the enamel surface. The inner layers of the biofilm exhibit higher fluoride concentrations than the outer layers. Overall, the fluoride content in the biofilm depends on both the salivary fluoride concentration and the plaque pH, with lower fluoride levels observed in biofilms at reduced pH values.

More than 99% of the total fluoride in the body is deposited in bones and mineralized dental tissues, including enamel, dentin, and cementum. The fluoride ion exhibits a remarkably high affinity for these mineralized tissues, with its incorporation exceeding that of Ca^{2+}. Fluoride is readily incorporated into hydroxyapatite crystals or binds to their surface. During amelogenesis, the incorporation of fluoride into hydroxyapatite occurs via heteroionic exchange, whereby hydroxyl groups in the hydroxyapatite crystals are replaced by fluoride ions from the blood, resulting in the formation of fluorohydroxyapatite.

In all mineralized tissues of the body, fluoride concentration is highest in the surface layers exposed to tissue fluids, which continuously supply this bioelement. The incorporation of fluoride into these solid tissues occurs in three distinct stages:

- the fluorine ions arrive in the hydration layer of the hydroxyapatite
- replacement of OH groups with F ions, on the surface of the hydroxyapatite crystals
- from the surface layers the fluoride ions arrive in the deeper layers of the solid tissues.

In this manner, fluorohydroxyapatite is formed, exhibiting lower solubility compared to hydroxyapatite. Furthermore, the incorporation of fluoride into hydroxyapatite crystals reduces the incorporation of other ions, such as carbonates, citrates, and magnesium, which collectively constitute the main “impurities” of enamel. The presence of these impurities increases enamel solubility in an acidic environment.

From the circulation, fluoride also reaches bone tissue, where it is readily incorporated. Bones serve as a major fluoride reservoir in the body and play a significant role in the biokinetics of this element. With advancing age, fluoride concentration in bone tissue increases. Importantly, fluoride is not irreversibly deposited in bone, allowing for its mobilization from these depots.

The concentration of fluorine in soft tissues is lower than the concentration of this element in blood plasma, except in the kidneys. Unlike hard tissues, the concentration of fluoride in soft tissues does not increase over the years, nor does it depend on the body's exposure to this element.

The concentration of fluoride in enamel (except in its most superficial layers), dentin, and cementum remains constant. These tissues do not participate in fluoride biokinetics, as they do not undergo remodeling like bone. Once incorporated, fluoride in dental tissues cannot be released. In enamel, fluoride concentration is twice as high in the surface layers and gradually decreases toward the enamel-dentin junction. In dentin, fluoride concentration is higher than in enamel, with the highest levels observed in the odontoblastic layer, immediately above the pulp. Cementum exhibits the highest fluoride concentration, even exceeding that of bone tissue. This is due to its minimal thickness, which exposes it more directly to fluoride-rich tissue fluids. Fluoride concentration in the inner layers of cementum is lower than in its outer layers.

Fluorine has the ability to pass through the placenta, from the mother's blood it reaches the blood of the fetus unhindered. In the fetus, fluorine is intensively incorporated into solid tissues during their development.

Fluoride is eliminated from the body through urine (60-70%), through feces (15%), through sweating, tears and breast milk (15%). The ionic fraction of fluoride is excreted through urine.

For a long time, it was believed that fluoride exerts its prophylactic effect against dental caries primarily through incorporation into hydroxyapatite crystals. During enamel development (amelogenesis), fluoride is incorporated into hydroxyapatite according to the principle of heteroionic substitution, in which the OH group from the crystals is replaced by fluoride from the blood, forming fluorohydroxyapatite (Figure 12.4). It was also assumed that topically applied fluoride on the enamel surface should remain in place long enough to allow substitution of the OH group by fluoride ions. However, more recent research indicates that fluoride embedded in hydroxyapatite—whether through systemic or local application—is not the primary mechanism of its preventive action.

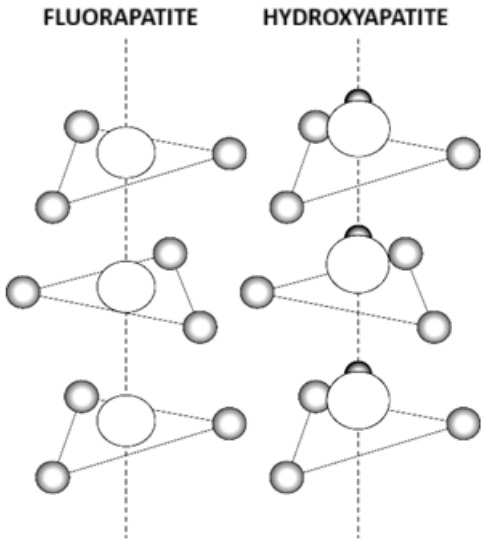

Figure 12.4: *Crystal structure of fluorapatite is more compact than the crystal structure of hydroxyapatite*

During local application (impregnation of the teeth with fluoride liquids or application of toothpastes with different concentrations of fluoride), small amounts of fluorides are applied, with the help of which the dental plaque is soaked with these anions. In both cases (systemic and local ingestion), certain effects are expected in the microenvironment of the plaque, which are significant for the maintenance of oral health (Figure 12.5).

The effects of fluoride in dental plaque are:

1. Fluoride inhibits the activity of the enolase enzyme, a key enzyme in the glycolysis process.

2. By inhibiting enolase, glycolysis—the primary catabolic pathway for all sugars—is halted.

3. With glucose metabolism blocked, many metabolic activities are disrupted, most importantly ATP production is impaired.

4. Blocking ATP synthesis prevents phosphorylation of glucose into glucose 6-phosphate, thereby hindering its use in both catabolic and anabolic pathways. Specifically, glycogenesis is inhibited, preventing bacterial glycogen synthesis.

5. Inhibition of glycogen synthesis stops the release of acidic metabolites, most importantly lactic acid.

Fluoride also inhibits heme-containing enzymes, including urease and catalase. This occurs when fluoride binds to the heme group, leading to inactivation of catalase. As a result, the bacterial cell's antioxidant defense is weakened, causing accumulation of free radicals and oxidative damage to cellular structures.

Additionally, fluoride is a potent non-competitive inhibitor of bacterial urease. Inhibition of urease disrupts the metabolism of nitrogen compounds within the bacterial cell.

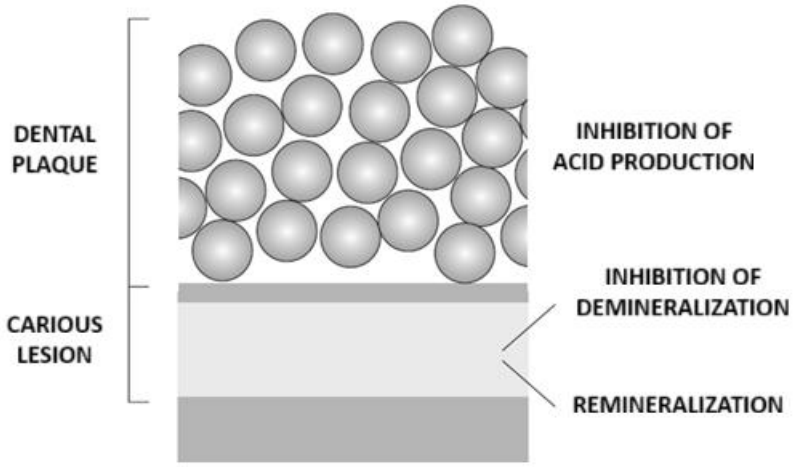

Figure 12.5: *Mechanism of action of fluorides. Fluorides inhibit acid production and solubility of hydroxyapatite crystals and stimulate remineralization.*

The local effect of fluoride is especially important, as the continuous intake of small amounts of this element provides the following benefits:

1. It promotes remineralization over demineralization, preventing the loss of mineral content in the enamel.
2. Upon local application, the following reaction occurs on the enamel surface:

$$Ca_{10}(PO_4)_6(OH)_2 + 2OF \rightarrow 10CaF_2 + 6PO_4 + H_2O$$

The released CaF_2 is readily soluble in water. However, in the oral environment, its solubility is reduced by phosphate ions, which facilitate the formation of fluoride deposits on the tooth surface.

3. When the pH in the CaF_2 depot drops below 5.5, dissolution occurs, releasing fluoride ions that help prevent enamel demineralization. These highly reactive fluoride ions bind to hydroxyapatite crystals, forming fluorapatite, which has lower solubility.

4. Increased local acidity can also lead to the release of HF (hydrofluoric acid) from CaF_2, which diffuses through the plaque layers and inhibits the enolase enzyme.

5. HF readily penetrates between enamel prisms and hydroxyapatite crystals in the deeper layers of enamel, where demineralization begins under the influence of H^+ ions. In these areas, heteroionic exchange occurs, forming fluorapatite, and HF reacts with released calcium ions to generate CaF_2. This deposited CaF_2 acts as a barrier against further demineralization, promoting remineralization and thus producing a therapeutic effect.

Considering both the effects of fluorides on the enamel surface and within dental plaque, the preventive and therapeutic significance of fluoride becomes evident.

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