

3 INVESTIGATION OF SALIVA

Topics to be reviewed:

Saliva, salivary composition and functions. Production and secretion of saliva. Buffering capacity, salivary buffering capacity. Organic components of saliva. Enzymes in saliva.

Pre-lab questions

1. Write the Henderson-Hasselbalch equation for a buffer solution and identify factors that determine the capacity of a buffer.
2. Give some examples of cariogenic compounds.
3. Write the equation for the reaction catalyzed by lactate dehydrogenase.
4. Give some examples of reducing disaccharides. Which test is commonly used to identify reducing sugar?

Introduction

Saliva is produced by the salivary glands located throughout the oral cavity. Most of the saliva is produced by the major salivary glands (glandulae parotis, glandulae submandibularis, glandulae sublingualis). Saliva consists of approximately 99 % water, containing a variety of ions (sodium, potassium, calcium, chloride, bicarbonate, phosphate) and organic components, represented by glycoproteins (mucins) responsible for lubrication and maintenance of salivary viscosity, enzymes (alpha-amylase, lysozyme and other), immunoglobulin A, lactoferrin and low molecular weight organic compounds. Concentrations of many analytes in saliva change with stimulation; are affected by salivary flow rate. The total volume of saliva produced each day in adults is from 0.5 to 1.5 L. Saliva performs many important functions in the mouth. These include mastication and initial digestion of food, maintenance of integrity of oral hard and soft tissues, antibacterial activity and remineralization of the teeth.

Saliva represents an attractive biological fluid for diagnostic use because of the noninvasive and relatively simple collection. Besides routine salivary investigation (determination of salivary secretion and buffering capacity, pH and enzymes), saliva is used for monitoring of hormone and medications level and detection of drugs and alcohol. Further determinations of microbial contamination (*Streptococcus mutans*, *Lactobacillus acidophilus*, *Candida albicans*) and the pH of dental plaque are performed for assessing the risk of dental erosion and caries.

3.1 Determination of salivary secretion rate

Different salivary glands produce saliva of different composition. The relative proportion contributed to the whole saliva by each of the gland pairs depends on the degree of stimulation. Thus, under resting conditions, basal secretion is produced mainly by the sublingual gland and smaller salivary glands, the unstimulated saliva is formed. After stimulation the stimulated saliva is produced mostly by glandula parotis and submandibularis. Salivary secretion is a reflex response and potent stimuli for salivation include the smell and the taste of food, thoughts of food, the presence and mechanical processing of food in mouth, mastication. Secretion of saliva is controlled by both sympathetic and parasympathetic nerves. Noradrenaline released from the sympathetic neurons activates β_2 -receptors of the acinar and ductal cells which stimulate production of high viscous saliva rich in mucins. The parasympathetic neurons release acetylcholine that interacts with muscarinic receptors on the acinar and ductal cells which resulted in production of serous, watery secretion.

Salivary secretion rate is measured under resting conditions or after stimulation with paraffin pellet. Salivary flow rate is defined as volume of saliva produced per time (ml/min). Because eating, drinking and medications affect salivary secretion rate, it is important to avoid eating and drinking, tooth brushing, and smoking for 1–3 hours before sampling.

Materials: Set Dentobuff Orion Diagnostica: Dentobuff test strips, paraffin pellets, disposable pipettes; test tubes, funnels, beakers, graduated cylinders, timer.

Procedure

a) Determination of unstimulated salivary secretion rate:

- ☞ Rinse mouth with drinking water.
- ☞ Sit in an upright, relaxed position with head tilted forward and allow saliva to pool in the mouth.
- ☞ Let saliva flow from your mouth through a funnel into the graduated cylinder or into beaker.
- ☞ Collect saliva for 15 min.
- ☞ Measure the volume of the saliva produced.
- ☞ Calculate the saliva secretion rate (ml/min).

b) Determination of stimulated salivary secretion rate:

- ☞ Sit in an upright, relaxed position
- ☞ Chew a paraffin pellet for 1 min. During this time, either spit out or swallow any saliva produced.
- ☞ Continue to chew the pellet for further 5 min during which any saliva produced is collected in a suitable vessel.
- ☞ Measure the volume of the saliva produced.
- ☞ Calculate the saliva secretion rate (ml/min).

► Evaluate the salivary secretion from the data given by the assistant.

Evaluation

The normal flow rate for unstimulated, resting whole saliva is 0.25 to 0.35 mL/min, low flow rate ranges from 0.1 to 0.25 mL/min and hyposalivation is characterized by a salivary flow of less than 0.1 mL/min.

The normal flow rate for stimulated whole saliva is 1 to 3 mL/min, low ranges from 0.7 to 1.0 mL/min and hyposalivation is characterized by a salivary flow of less than 0.7 mL/min.

The fact that the volume of saliva secreted and its composition vary greatly among individuals and in response to the several stimuli, the circadian rhythm, the degree of hydration and further factors, should be taken into account in the course of evaluation. The evaluation of stimulated and unstimulated salivary secretion rate should be made from repeated measurements and follow-up.

Salivary secretion dysfunction may be characterized by either **hypersalivation** or **hyposalivation**. **Hypersalivation** (increased salivary flow) is the most often triggered as a reflex response, for example in the course of a dental treatment. Increased salivation (ptyalism) can be caused also by some medications (pilocarpine).

Hyposalivation (decreased salivary flow) occurs in the case of dehydration, long-term taking drugs containing atropine, radiotherapy involving main salivary glands and in case of Sjörger's syndrom. **Xerostomia**, the subjective sensation of dryness in the mouth, may result from the reduced salivary flow. Xerostomia can lead to an increased risk of dental cavities, infections in the oral cavity and difficulties in chewing, swallowing, and speaking. Several drugs, such as tricyclic antidepressants, antihistamines, can cause xerostomia.

3.2 Estimation of salivary buffering capacity

Salivary buffering capacity is important for elimination of acids in the mouth and its estimation is an indicator of the risk of dental erosion and caries. High buffering capacity prevents dental caries development, while a low buffering capacity is strongly associated with increased erosion. Saliva contains three buffer systems: hydrogen carbonate, hydrogen phosphate, and protein buffer system. Hydrogen carbonate is the major buffer of saliva. Normal salivary pH is 5.6 - 7.9. The buffer capacity depends on the ratio of both buffer components, their total concentrations, and also the pK_a value of buffer acid.

The standard method to determine the saliva capacity is the Ericsson's test. The method is based on the measurement of the salivary pH by a pH meter after a certain amount of HCl is added into collected stimulated or unstimulated saliva, followed by a waiting period of 20 min for the elimination of carbon dioxide. Commercial strip tests represent a new and simplified chair-side method to estimate the salivary buffer capacity.

The Dentobuff[®] Strip is used for estimation of buffering capacity in stimulated saliva. The strip consists of a pH indicator paper that has been impregnated with acid. When a drop of collected saliva is added to the test pad of the strip, a reaction of salivary buffer with acid present in the test pad occurs. An pH indicator system incorporated in the test strip changes colour, clearly showing the buffer capacity of the saliva that depends on the presence and concentration of buffering systems in saliva.

Materials: Set Dentobuff Orion Diagnostica: Dentobuff test strips, paraffin pellets, disposable pipettes, test tubes, funnels, beakers, graduated cylinder, timer

Procedure

☞ Collect stimulated saliva as described in task 3.1.

Buffer capacity is commonly taken simultaneously with the secretion rate. Use saliva collected in task 3.1.

☞ Apply one drop of stimulated saliva to the pH pad using a disposable pipette.

☞ After 5 min, compare the colour of the pH pad with the colour chart.

Evaluation

The method differentiates stimulated saliva with low, intermediate and high buffering capacity. High buffering capacity of saliva is essential for effective protection of teeth against acids produced by bacteria present in the mouth.

Buffering capacity of stimulated saliva according to the Dentobuff[®] Strip

The colour of the pH pad	The final pH of saliva	Buffering capacity
Blue	≥ 6	High
Green	4,5-5,5	Intermediate
Yellow	≥ 4	Low

Viscous saliva may cause the colour reaction to be uneven or mixed. In such cases, buffering capacity should be evaluated according to the colour indicating the lowest value.

The test should be performed as soon as possible after saliva stimulation. To avoid daily variation, samples from the same patient should be taken at the same time of day during follow-up. Hydrogen carbonate is the major component of salivary buffering capacity. The hydrogen carbonate concentration, in turn, is strongly dependent on salivary secretion rate.

3.3 Determination of organic compounds in saliva

Saliva contains several low molecular weight compounds, including urea, creatinine, uric acid, glucose, and amino acids. Concentrations of many analytes vary in response to salivary flow rate, some stimuli, and collection method.

Carbohydrates played an essential role in the pathogenesis of dental caries. Some sugars, for example sucrose, glucose, and fructose are readily fermented by bacteria *Streptococcus mutans* and *Lactobacillus* species found in the mouth. The acids produced by these bacteria (mainly lactic acid, but also some formic and acetic acids) reduce the pH of the mouth to less than 5.0 at which demineralization of enamel occurs and dental decay begins.

The glucose concentration in saliva is actually very low (about 0.05 mmol/l) at normal conditions. A slight increase in salivary glucose levels is associated with diabetes. Lactate is generally present in saliva in trace concentrations. Slightly higher salivary lactate levels are observed after consumption of diet rich on sugars and insufficient dental hygiene.

3.3.1 Reducing properties of glucose

Glucose containing free hemiacetal (anomeric) hydroxyl group is able to reduce even relatively weak oxidizing agents, e.g. Benedict's reagent. Therefore, it is called as a *reducing sugar*. All monosaccharides and oligosaccharides containing free anomeric hydroxyl group are reducing sugars.

Reaction with Benedict's reagent can be used as a non-specific test for glucose and other reducing compounds in saliva. It is also used to detect reducing substances (glucose) in urine. The specific test for glucose is based on enzymatic method with glucose oxidase and peroxidase (Task 1.2.1.).

Materials: Benedict's reagent (prepared by dissolving 0.7 mol of Na_2CO_3 and 0,6 mol trisodium citrate in 700 ml demi-water with heating. After cooling, a solution containing 70 mmol CuSO_4 is added and made up to 1 L with water), solutions of D-glucose, saccharose (approx. 10 g/l), saliva, plastic Pasteur pipettes, test tubes, beaker.

Procedure

- ☞ Add only -1-2 drops of tested sample (saccharide or saliva) to about 1 ml Benedict's reagent, mix gently. (Recommended ratio of the reagent and sample must be maintained)
- ☞ Place all of the test tubes in the boiling water bath for 2 minutes.
- ☞ Use following samples:
 - a) solution of D-glucose
 - b) solution of sucrose
 - c) saliva (use saliva collected in task 3.1, and processed through filtration paper).
- ☞ Record and explain the results .

► Write the equation of reaction of β -D-glucopyranose with Benedict's reagent (i.e. Cu^{2+} ions in alkaline medium in the presence of citrate) Products of the reaction, i.e. cuprous oxide Cu_2O (red) and cuprous hydroxide CuOH (yellow) are insoluble.

3.3.2 Determination of lactate in saliva

Lactate oxidase catalyses the oxidation of lactic acid to pyruvate and hydrogen peroxide. Peroxidase then catalyses the reaction of hydrogen peroxide with N-ethyl-N-(2-hydroxy-3-sulfopropyl)-m-toluidine (TOOS) and 4-aminophenazone to form blue-violet product. Color intensity of product measured at 550 nm is proportional to the lactate concentration in the sample.

Materials: Set Lactate Biovendor: Reagent-lactate: lactate oxidase 3,3 $\mu\text{kat/L}$, peroxidase 16,7 $\mu\text{kat/L}$, TOOS 0,3 mmol/l, 4-aminophenazone 0,1 mmol/L, Calibrator-lactate (concentration is given on the label), saliva, test tubes, pipettes.

Procedure

- ☞ Pipette 0,25 ml of reagent into two test tubes.
- ☞ Add 10 μl of calibrator-lactate into the first tube.
- ☞ Add 10 μl of saliva into the second tube. Use saliva prepared in task 3.1. and processed through a filtration paper.
- ☞ Mix both test tubes and incubate for 20 minute at room temperature (or 10 minutes at 37 °C)
- ☞ Record and explain the appearance of test tubes.

3.4 Detection of α -amylase and its substrate specificity

Salivary α -amylase (ptyalin) belongs to the most important enzymes occurring in saliva and can comprise up 50 % of protein content in saliva. Salivary α -amylase is formed mainly in glandula parotis (about 80 %) and less in glandula submandibularis (20 %). Alpha-amylase hydrolyses α -1,4-glycosidic bond of starch into breakdown products as dextrin, maltose, maltotriose. The products of enzymatic hydrolysis can be detected by Benedict's reagent that is used as a test for the presence of reducing sugar (see task 3.3.1).

Materials: Starch and sucrose (about 10 g/L), saliva, Benedict's reagent (prepared by dissolving 0,7 mol Na_2CO_3 and 0,6 mol trisodium citrate in 700 ml demi-water with heating). After cooling, the solution containing 70 mmol CuSO_4 is added and the volume made up 1000 ml), test tubes, plastic pipettes

Procedure

- ☞ Prepare two test tubes.
 - ☞ Pour about 1 ml of substrate-starch to the first test tube and 1 ml of substrate-sucrose to the second test tube.
 - ☞ Add 0,1 ml of saliva to both test tubes. Use saliva collected in task 3.1. Use saliva prepared in task 3.1. and processed through a filtration paper.
 - ☞ Shake both test tubes and incubate 20 minute at the room temperature (or 10 minutes at 37 °C)
 - ☞ Perform Benedict's test for detection of reducing sugars in both test tubes – see task 3.3.
 - ☞ Evaluate the results of Benedict's test in both test tubes.
 - a) substrate - starch:
 - b) substrate – sucrose:
- ▶ Record and explain obtained results and characterize substrate specificity of α -amylase.

Other enzymes in saliva

Saliva shows relatively high activity of enzymes. Besides digestive enzymes (α -amylase, lingual lipase) human saliva also contains enzymes participating in the protection of oral cavity and organism, as lysozyme, peroxidases, superoxide dismutase. The other several minor enzymes with specific functions, as alcohol dehydrogenase, are found in saliva.

Under pathological condition, for instance during periodontal diseases, many enzymes (as elastase, collagenase, proteinases) involved in tissue degradation processes, can be detected or intracellular enzymes released from the damaged cells of the tissues can be determined. Enzyme assays in saliva appear to be good way to supplement microbial tests. Some of enzymes, as AST, ALT, LD, ALP, belong to enzymes usable for early diagnosis of periodontal diseases.

Examples:

Lactate dehydrogenase: Physiological values of LD catalytic concentration in saliva are approximately comparable with the values in serum. Elevated levels of LD are related to damage of periodontal tissues. (Note: Reference values of LD catalytic concentration in serum are for adults 3,8-7,7 μ kat/L)

Alkaline phosphatase: Physiological values of ALP catalytic concentration in saliva are approximately comparable with the values in serum. Elevated levels of ALP in saliva are connected with inflammatory processes in oral cavity and with destruction of periodontal tissues. (Note: Reference values of ALP catalytic concentration in serum are for adults 0,7–2,2 μ kat/L, for children up to do 8 μ kat/L).

It is necessary to take into consideration that the results of evaluation of catalytic activity of enzymes are varied and are affected by volume of saliva, rate of secretion of saliva, stimulation and obtained results in patients must be always compared with healthy population.