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Laboratory Exercises

Human Salivary α-Amylase (EC.3.2.1.1) Activity and Periodic Acid and Schiff Reactive (PAS) Staining

A USEFUL TOOL TO STUDY POLYSACCHARIDES AT AN UNDERGRADUATE LEVEL

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Health science education is presently in discussion throughout Europe due to the Bologna Declaration. Teaching basic sciences such as biochemistry in a health sciences context, namely in allied heath education, can be a challenging task since the students of preclinical health sciences are not often convinced that basic sciences are clinically valuable (J. R. Rudland, S. C. Rennie (2003) The determination of the relevance of basic sciences learning objectives to clinical practice using a questionnaire survey, *Med. Educ.* (*Oxf.*) 37, 962–965; E. C. Wragg (2003) How can we determine the relevance of basic sciences learning objectives to clinical practice?, *Med. Educ.* (*Oxf.*) 37, 948–949). Thus, nowadays teachers are compelled to use their imagination to be able to elaborate laboratory sessions aiming for the understanding of theoretical concepts that are also clinically related: in other words, basic concepts and skills that underlie the competencies demanded of the future health professional. In the present work, we describe a set of laboratory sessions implemented in the discipline of biochemistry, belonging to the first year of several courses of allied health professionals, which can also be implemented in other health sciences courses. These sessions focus on the characteristics and properties of carbohydrates. The exercises we propose include two different laboratory practical sessions based on a histopathological routine technique known as periodic acid and Schiff reactive that is currently used to detect sugar metabolic and tumor diseases (J. M. T. Rivera, C. T. López, B. C. Segui (2001) *Bioquímica Estructural: Conceptos y Tests*, Tebar Flores, Madrid). The methodology described enables the demonstration of some biochemical properties of polysaccharides, namely animal and vegetable, and the catalytic activity of the human salivary α-amylase (EC.3.2.1.1) enzyme. A further comparison between α-amylase activity *in vitro* and *in situ* is also possible by the proposed methodology. Additionally, to this extent, a comparison between the results of the learning improvement that occurred after the implementation of this tool is presented.

Keywords: Biochemistry, carbohydrates, α-amylase, histochemistry.

GENERAL INTRODUCTION

One of major problems faced by biochemistry teachers is the choice of practical laboratory sessions that can narrowly follow the theoretical classes [5, 6]. Additionally, pedagogical laboratory sessions should be simultaneously rapid, simple, non-expensive, and clinically correlated [1, 2]. In the present work, we developed two laboratory sessions specifically aimed toward biomedical students and health allied sciences students that will be centered on carbohydrate properties and the study of enzymatic activity. The sessions included two different practical classes based on a histopathological routine technique known as periodic acid and Schiff reactive (PAS)[1] staining. The PAS technique is used, normally, as a routine staining procedure for liver and kidney biopsies to visualize basal membranes, fungus, secreting adenocarcinoma from undifferentiated squamous cell carcinoma, and mucosubstances secreted from the epithelia of various organs, among others [7–10].

In the proposed laboratory sessions, the PAS technique was used without or after digestion with α-amylase (EC.3.2.1.1). The combination of those two approaches

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[1] The abbreviations used are: PA, periodic acid; PAS, PA/Schiff reactive; BSA, bovine serum albumin.

allowed the demonstration of biochemical properties of polysaccharides and the study of the catalytic activity of human salivary α-amylase. The procedure developed also permitted the comparison of α-amylase activity *in vitro* and *in situ*.

The staining mechanism of the periodic acid and of the colorimetric properties of the Schiff reactive was explored in the first laboratory session. Furthermore the activity of α-amylase in the context of the PAS reaction *in vitro* on several substrates, such as water, D-glucose, starch, bovine serum albumin (BSA), and cholesterol, was shown [11]. In the second laboratory session, the PAS staining of several histological thin sections was undertaken to distinguish the presence of polysaccharides and their different distribution on organs and tissues [7].

The main concepts underlying the present work and the proposed procedure are described below. Examples of the results obtained in each session are also presented in the following sections.

FIRST SESSION, STUDY OF α-AMYLASE ACTIVITY USING PAS STAINING

Introduction—Carbohydrates are the primary source of energy in our diet. They are composed of carbon, hydrogen, and oxygen. The simplest forms are the monosaccharides, of which glucose holds prime importance [11–14]. Two monosaccharides may join to form a disaccharide. The polymeric combination of many disaccharides gives rise to polysaccharides. Glycogen is an important energetic storage compound in animals, and its structure is similar to starch, the main glucose storage compound of plants. Starch is a compound formed by amylose and amylopectin. The structure of amylopectin and glycogen is similar. Amylose has a linear structure with D-glucose residues linked with α(1→4) glycosidic bonds. Amylopectin and glycogen are branched polysaccharides of D-glucose residues with a linear structure maintained by α(1→4) bonds and also branched regions with α(1→6) bonds [11–15].

Starch is a dietary essential compound, and its degradation begins in the mouth. This degradation is achieved by enzymatic hydrolysis of salivary amylases. There are three groups of amylases, α-amylases (EC.3.2.1.1), β-amylases (EC.3.2.1.2), and γ-amylases (EC.3.2.1.3). In animals, α-amylases are present in saliva and pancreatic secretions, but they can also be found in plants, fungi, and bacteria [13, 16]. They are endoenzymes randomly acting in inner α(1→4) glucose bonds producing besides α-maltose, α-maltotriose, D-glucose, and also α-dextrin, which contains α(1→6) bonds. This activity is called diastase, and it was first described by Payen and Person in 1883 [13].

The α-amylases with known three-dimensional structures are multidomain, single polypeptide chain proteins. Besides an α_8/β_8-barrel, they all comprise an antiparallel β-stranded domain. The active site is localized in the cleft of the α_8/β_8-barrel domain. All α-amylases are metalloenzymes containing at least one calcium ion per enzyme molecule, which is essential for activity and stability [3, 14].

Objectives—The main objective of this session is to identify the presence of polysaccharides by PAS staining and to understand the mechanism underlying this procedure in the presence or absence of α-amylase. For that purpose, a set of three tube series containing different molecules such as water, BSA, starch, D-glucose, and cholesterol was used. Since polysaccharides give a positive response to PAS reaction, this staining procedure is often used on a variety of plant and animal material to detect the presence and intracellular localization of polysaccharides [17]. The Schiff reactive is the main component of this test. It binds to aldehydes in insoluble substrates, forming a purple compound. Periodic acid is an oxidizing agent that breaks the C–C bond between two adjacent hydroxyl groups. The 1,2-diol group in glucose is converted into a dialdehyde (Fig. 1). The advantage of periodic acid (PA) treatment lies in the specificity of its oxidation. It forms aldehydes within the polysaccharide molecule, but it does not continue the oxidation of polymers of low molecular weight water-soluble forms, meaning that this oxidation preserves glycosidic linkages. Thus, polysaccharides, containing dialdehyde groups after PA treatment, maintain their insoluble forms, which might react afterward with Schiff's aldehyde reagent to form a purple colored product [4, 9].

Materials and Methods—Three sets of five similar tubes, labeled A, B, and C, were prepared as follows: distilled water, BSA solution (Sigma), starch solution, D-glucose (Merck) solution, and cholesterol (Merck). All solutions were prepared as described before [11]. PA purchased from Panreac was prepared in a 10% solution (v/v); α-amylase was purchased from Sigma, and it was prepared in a solution containing 2.5 mg/ml and stored at 4 °C. The Schiff reactive was purchased from Micron and used as directed by the manufacturer.

In the first series, five drops of the Schiff reactive were added and were allowed to act for 5 min. In the second series, a treatment of 1 ml of PA-containing solution was made 10 min before the staining with Schiff dye. Finally, in the third series, the procedure of the second series was repeated. However, in this case, 500 μl of α-amylase solution was added to the tubes before the addition of PA and Schiff reactive. This incubation was performed for 20 min in a 37 °C prewarmed chamber.

Results—All the results obtained by students were summarized in a table as exemplified in Table I. As expected, starch solutions from series two (Fig. 2*B*) presented the unique positive result. All the other tubes showed a negative response. This experiment demonstrates the high affinity of PAS to insoluble polysaccharides of high molecular weight [4, 7, 9, 11]. By comparing the starch-containing tube of the second series with the starch solutions from the other two series, remarkable differences could be observed once those starch solutions also presented a negative response to Schiff. Those differences are useful to explain the PAS stain after or before diastase and also the specific hydrolytic activity of PA. On one hand, the starch-containing tube from the first series could not present a positive response to Schiff once there were not sufficient free aldehyde groups. On other hand, the starch-containing tube from series three could not give a positive response to PAS once α-amylase digested the insoluble polysaccharides of high molecular weight into low molec-

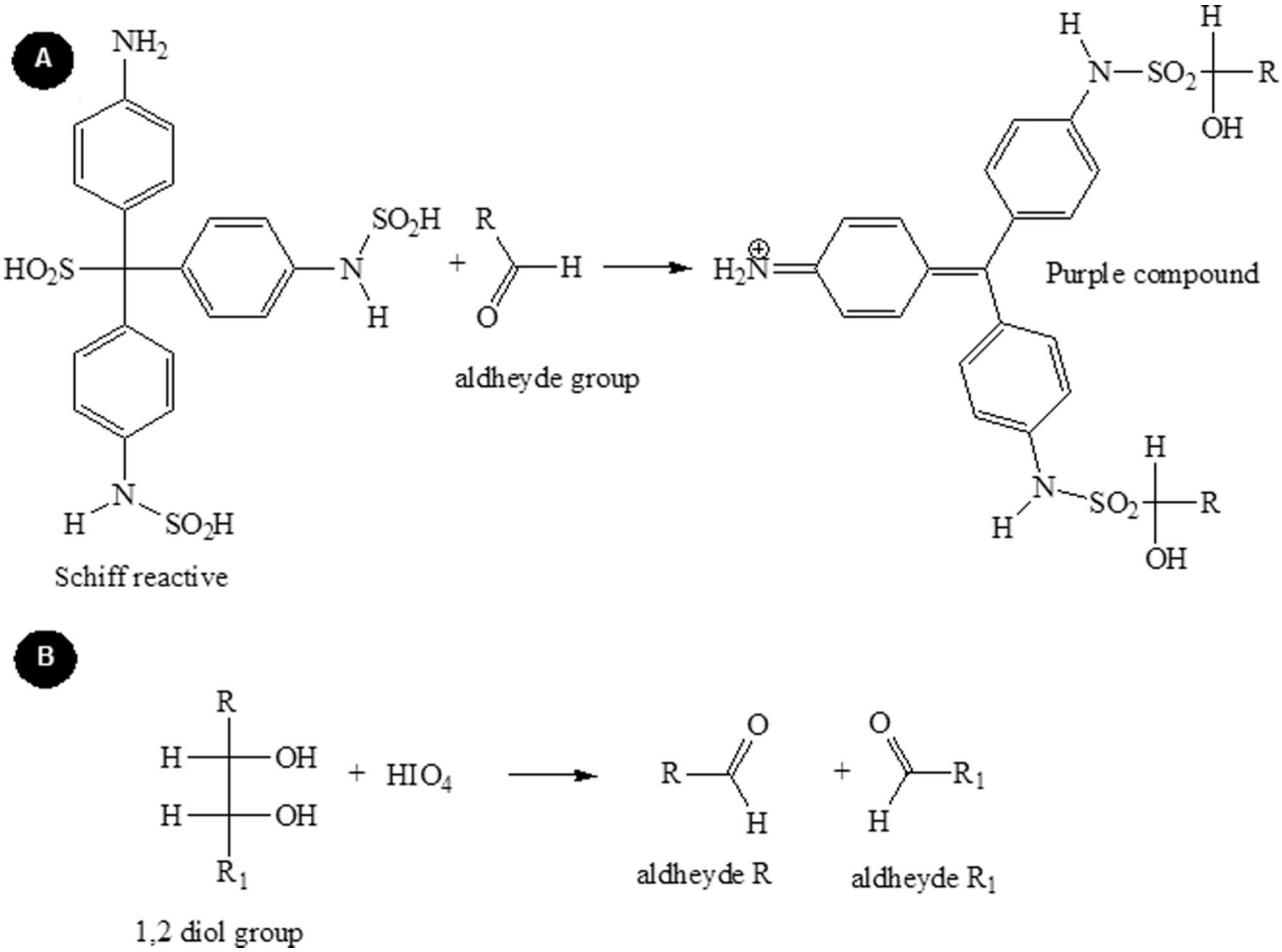

FIG. 1. **PAS mechanism.** *A*, Schiff dye reacts with aldehyde groups to form a purple compound. The Schiff reactive may be used to detect polysaccharides, especially after a pretreatment with PA. *B*, PA will recognize 1,2-diol groups within polysaccharidic molecules and will oxidize then into a dialdehyde compound.

TABLE I
PAS test results of color development after PAS staining in vitro

Series	PAS color development				
	Water	BSA	Starch	D-Glucose	Cholesterol
Schiff	Pink	Pink	Pink	Pink	Pink
PA + Schiff	Pink	Pink	Purple	Pink	Pink
α-Amylase + PA + Schiff	Pink	Pink	Pink	Pink	Pink

ular weight sugars that have no affinity to this enzyme.

SECOND SESSION, COMPARISON OF α-AMYLASE ACTIVITY ON LIVER AND INTESTINE POLYSACCHARIDES

Introduction

In the last century, the Schiff reactive has been modified by McMannus and started to be used in pathology [4, 9]. Periodic acid and Schiff reactive technique has since then become broadly used in histochemistry/cytochemistry studies to identify carbohydrates such as the glycogen and mucopolysaccharides. PAS histological technique can be performed with and without diastase. The glycogen can be found physiologically in cardiac and skeletal muscles, skin, parathyroid glands, and especially in liver [15].

Objectives

The main purpose of this session is to understand the localization and anatomical distribution of high molecular weight polysaccharides and also be able to distinguish which of those may be glycogen or another more complex polysaccharide. To accomplish this, we propose an approach that uses mice liver and intestine fragments embedded in paraffin blocks. Slides of thin sections of this material were then prepared to stain with PAS or PAS after diastase with salivary α-amylase to distinguish between glycogen present in liver and other polysaccharides such as mucopolysaccharides present in goblet cells of intestine [7]. Mucopolysaccharides or glucosaminoglycanes are the general designation for all polysaccharides composed of alternating units from uronic acids and glycosamines [18].

Materials and Methods

Thin Sections—Fragments of liver and intestine were dissected from mice and fixed in paraformaldehyde 4% for 16 h and then dehydrated in a series of ethanol at 50, 70, 80, 95, and 100% for 1 h each with agitation. After that,

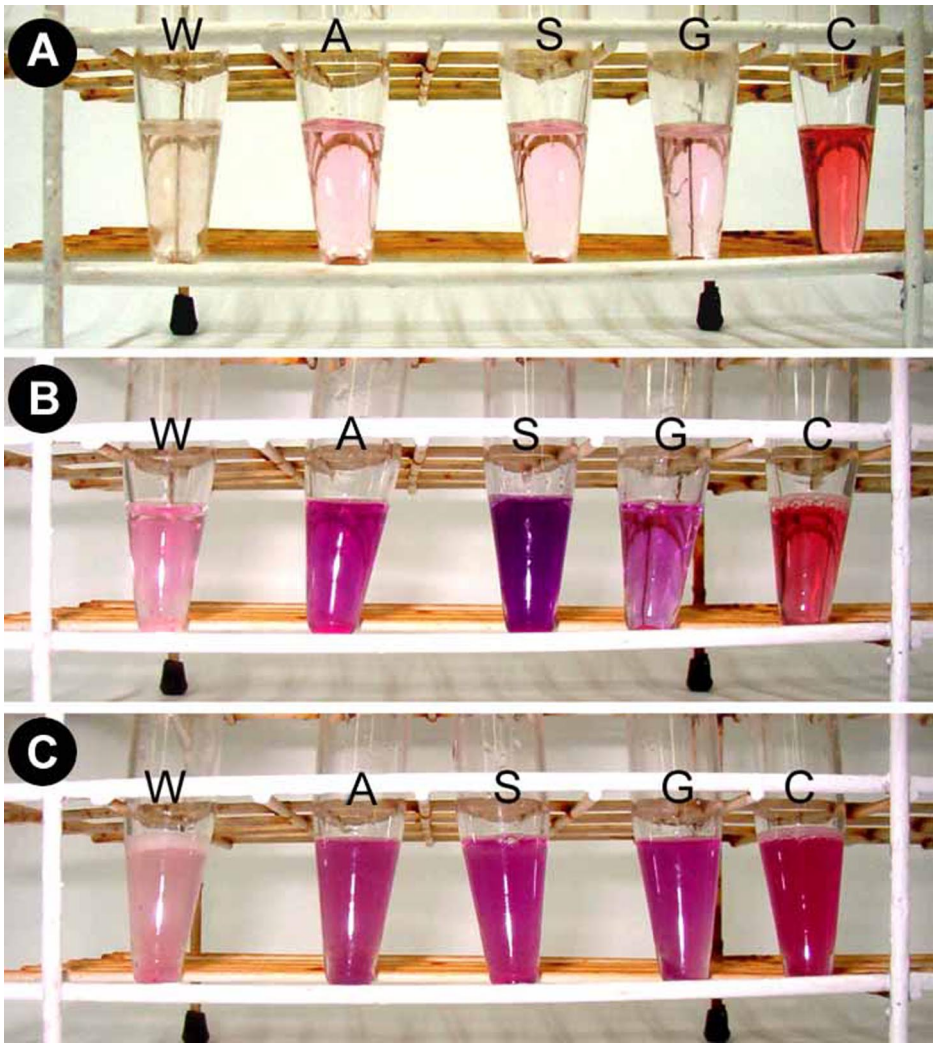

FIG. 2. **Color development of PAS dye.** The legend is as follows: *W*, water; *A*, bovine serum albumin; *S*, starch; *G*, glucose; and *C*, cholesterol. *A*, solutions were treated only with Schiff dye. *B*, solutions were pretreated with 10% periodic acid and then with the Schiff reactive. *C*, solutions were digested with α-amylase, following the same procedure as in *panel B*.

ethanol was changed by xylene (2 h), and it was impregnated with a solution of xylene-paraffin for another 2 h at 56 °C. A single block of paraffin was made containing both types of tissues. Thin sections were prepared at 4–5 μm in a microtome and attached to a glass slide. Slides preparations were warmed at 60 °C before being stored or used. Afterward, tissues were deparaffinated in xylene (30 min) and rehydrated in a decreasing series of alcohols at 100, 80, and 70% for 10 min each under running water.

Chemicals—The Schiff reactive, α-amylase solution, and PA were purchased and prepared as described before under "Material and Methods" for the previous section. Xylene, ethanol, hematoxylin (modified to Gill III), and Neo-mount (mounting medium) were all purchased from Merck.

Procedure—Two slides per group were distributed; each slide contained both liver and intestine samples and were designated slides number 1 and number 2. Slides number 2 are the experimental control, whereas slides number 1 were submitted to digestion with α-amylase.

The slides were first deparaffinized in xylene for 10 min, and this step was repeated three times at room temperature. Deparaffination may also be achieved by a single overnight step on xylene just the day before the class. This was followed by rehydration in a decreasing series of ethanol at 100, 80, 70, and 50% for 10 min each. Then the slides were washed for 5 min in running water and rinsed in distilled water. The α-amylase solution was then dropped over slide number 1 abundantly to cover all the slide glass followed by incubation at a 37 °C for 20 min. Finally, slide number 1 was rinsed in distilled water after the incubation with the enzymatic solution. Slide number 2, the control, was incubated in distilled water for the same period of time. Then both slides were oxidized in the presence of PA for 10 min and rinsed in distilled water after that. Differential staining of polysaccharides by Schiff's reagent was then performed submitting both slides simultaneously for 10 min to this dye. Staining was followed by a washing step in running water for 10 min. (This intensifies the color reaction.) Finally, the nuclei were contrasted with hematoxylin for 5 min, and then the excess of dye was washed in running water for 10 min. Dehydration in ascending alcohols series (70, 80, 100, and 100%) precedes clearing (xylene) and mounting with a resinous medium (Neo-mount). The slides were finally observed by students using a light microscope. The observations allowed the students to complete Table II.

RESULTS

An example of the results obtained with the proposed approach is present in Table II and further shown in Fig. 3. The α-amylase activity was observed in glycogen in the liver sample (Fig. 3, *A* and *B*) since a negative response to

PAS staining was obtained after diastase but not in its absence. This result is not surprising because it is known that α-amylase activity hydrolyzes $\alpha(1\rightarrow4)$ glycosidic bonds between glucose residues [16]. However, as expected, in intestine tissues, the PAS staining, with or without α-amylase digestion, does not show any differences (Fig. 3, C and *D*). This is due to the nature of the molecules stained. The intestine molecules stained by PAS are glucosaminoglycanes, which are not a homopolymeric structure of glucose, bonded by $\alpha(1\rightarrow4)$ glycosidic bonds, but a polymeric complex structure. Thus the present approach is also useful not only for the distinction of several types of polysaccharides but also to understand its distribution in different animal tissues.

TABLE II
PAS test results of color development after histochemical PAS staining

Tissue	PAS color development	
	No diastase	With diastase
Liver	Positive purple substances	Negative pink background
Intestine	Positive purple substances	Positive purple substances

GENERAL DISCUSSION

The development of new teaching strategies will probably allow a higher effectiveness in the acquisition of com-

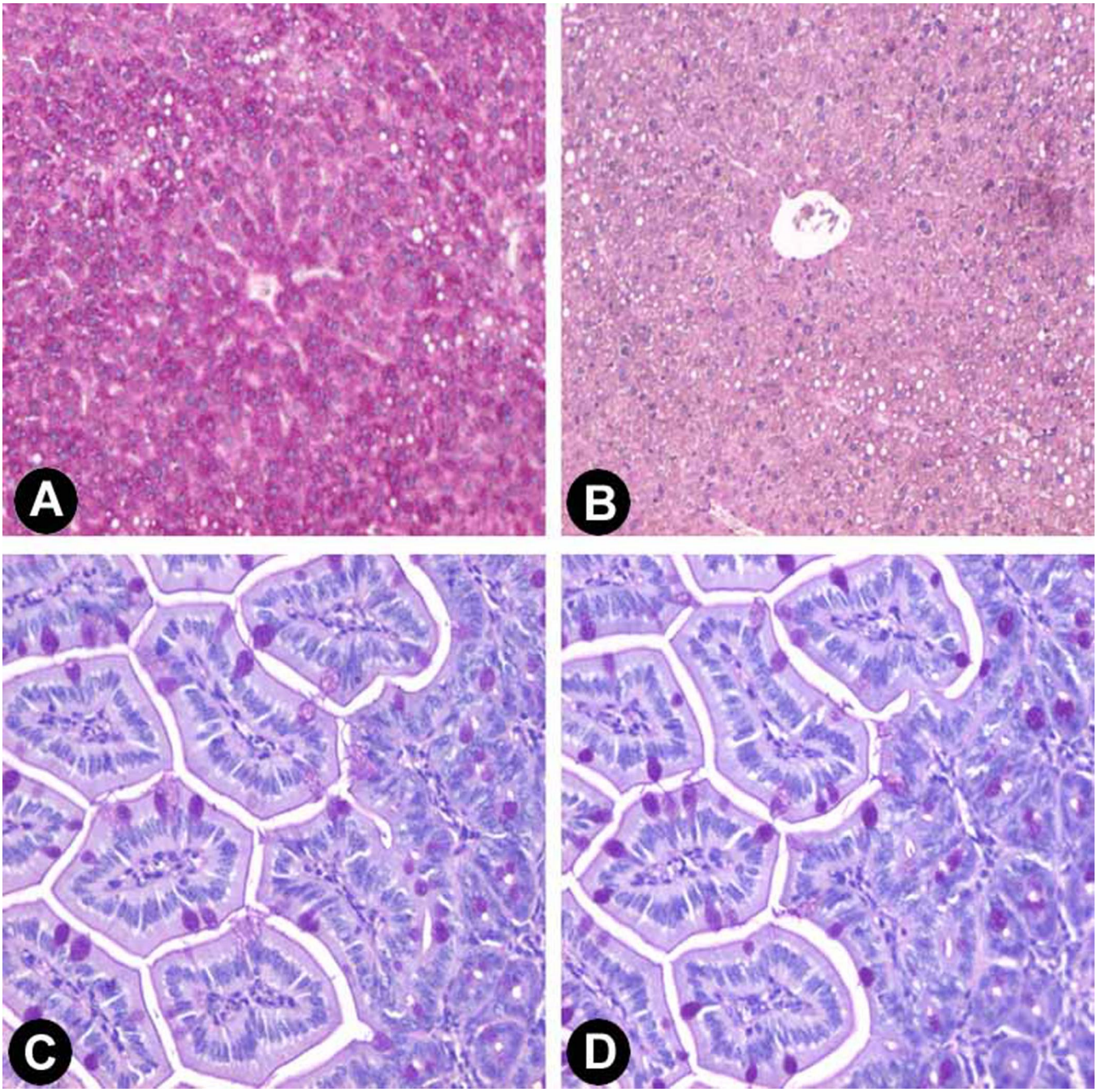

FIG. 3. **Microphotography of liver (*A and B*, PAS, ×120) and intestine (*C and D*, PAS, ×360) mice tissues.** *A*, a strong color was due to glycogen staining in the liver sample. *B*, weak color developed after diastase and hydrolysis of glycogen in the liver sample. *C*, since goblet cells produce mucopolysaccharides, they are PAS-positive, as shown by its strong staining. Nevertheless, these goblet cells are resistant to α-amylase since its color is not affected by this enzyme activity, as shown in *D*.

petencies since memory storage and retrieval, cognition, and learning can be enhanced if properly stimulated [19]. It is important to affirm that these types of experiences enhance the learning within the learning structures of the student. As Canário defends, "There can be no learning if not *with* and simultaneously *against* the previous knowledge of the individual, in the sense that learning (the accommodation of a equilibration structure) supposes, at minimum, that there is, at the same time, assimilation of a new information by an equilibration structure as well as conflict between both." [20].

To introduce educational improvements, a questionnaire was designed to evaluate the opinion of first year students enrolled in the program concerning the main aspects of the discipline in two consecutive years, before and after the implementation of this tool. Students were asked about the level of their approval of the organization of the discipline, the role of the teaching staff and pedagogical quality, the professional relevance of the program, the time/knowledge fit, and the educational media. The aim was to identify problems and to introduce changes that might improve both the program and the performance of the teaching staff and to also achieve awareness toward the need for multiprofessional education. These results, as a whole, were taken into account in the planning of the following year's programs. Among the data collected, the need for increasing practical sessions with applications for future allied health professionals was strongly pointed out. Through the content analysis of the open answer questions of the above mentioned questionnaires, we verified that in the year before the implementation, 14.3% of the students pointed out the need for more practical sessions directed toward their professional competences, whereas in the year after, only 4.4% of the students pointed out the same need. Concerning the professional relevance of the contents and practical sessions of this course, in the year before the implementation of this new tool, the students' opinion average was 3.92 (in a five-point Likert scale) with an S.D. of ± 0.36, whereas in the year after, the average presented was 4.20 (in a five-point Likert scale) with an S.D. of ± 0.45.[2] The above mentioned results bring into focus an improvement of the satisfaction of the students as well as an improvement of the professional relevance of these practical classes, although there was only a slight increase in the marks obtained in the biochemistry discipline. In fact, the average mark obtained before the implementation of this tool was 13.3 (out of 20) with an S.D. of ± 1.76; the average mark obtained after the implementation of this tool was 13.8 (out of 20) with an S.D. of ± 1.16.

In the present work, a simple and easy method to learn about polysaccharides and α-amylase activity is proposed. Among all the advantages of the applications that this method can allow for, some need to be mentioned. (i) The great simplicity of the PAS staining; (ii) all the reactives are very cheap and easily obtainable; (iii) if there are no opportunities or means to prepare the solutions, they are all commercially available at low cost, and there is no special recommendation for any component or any distributor preference; and (iv) whenever instructors want to apply *in vitro* approaches, the material required is very simple as well as mandatory in any biochemistry laboratory.

The histochemical approach may need further collaboration from a histology laboratory. Nevertheless, we strongly recommend a strict collaboration between departments to offer a multidisciplinary vision to students. In our particular case, students are learning histology at the same time that they are learning biochemistry. We believe that understanding the function of biomolecules and their distribution over the human body will reinforce learning, memory, and comprehensive knowledge of tissue composition, architecture, and function.

[2] C. Prudêncio and R. Fonte, submitted for publication.

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