INSTRUCTOR GUIDE

Experiment 701. Enzyme Cytochemistry

I CONTENTS OF THE CHEMICAL PACKAGE

	Quantity
Transfer pipets	10
Tetrazolium	1 ml
Iodine Solution	1 ml
Hydrogen Peroxide	1.3 ml
Chloronapthol	5 ml
Tris Buffer	5 ml
Corn Seeds	1 pack
Onion Bulbs	1
Chicken Blood Smears	8
Methylene Blue (.05%)	1 ml
Eosin (.2%)	1 ml
LDH-Substrate	1 tube
Nonidet P-40	1 ml
Dishes for slide incubation	3

II Preparation of Solutions

Distilled or deionized water is required to prepare the solutions listed below. You will also need to provide ethyl alcohol. All stock solutions except the chloronapthol should be warmed to room temperature before dilution. The blood smears should be stored in the refrigerator while the other chemicals should be stored in the freezer.

Tetrazolium - Add 1ml of the Tetrazolium concentrate to 25ml of water.

Iodine Solution - The tube provided with the Chemical Package contains 1 ml of a concentrated solution. Add the 1ml to 99ml of water and store in a tightly sealed bottle in the refrigerator.

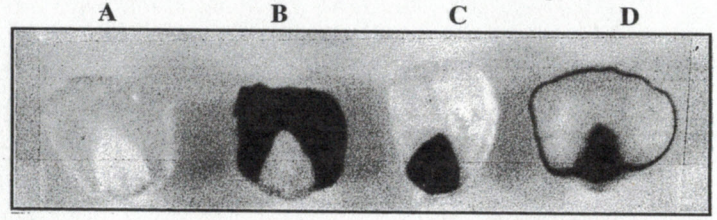
Peroxidase Substrate - The solution should be made immediately before the exercises in Part A and B. In each case, add the following to 20ml of water: 0.5ml chloronapthol, 0.2ml hydrogen peroxide (H_2O_2) and 0.6ml of Tris Buffer. The three stocks (Tris, H_2O_2 and chloronapthol) should then be returned to the freezer.

Nonodet P-40- Add the 1ml of the nonodet P-40 to 50ml of water.

LDH-Substrate - To prepare the solution, transfer the contents of the LDH-Substrate tube to 50ml of distilled water and stir for a few minutes until the powder is dissolved. The solution should be protected from light as much as possible and used immediately after preparation. After preparation, place 15ml of the substrate solution into 3 of the plastic dishes. Do not mouth pipet this solution.

III EXPERIMENTAL RESULTS

Part A: Location of starch, peroxidase, and sites of respiration.



A = water
B = Iodine
C = Tetrazolium
D = Peroxidase Substrate

Component/Area Stained
Starch/Endosperm
Respiration/Embryo
Peroxidase/Aleurone (Lower activity found in embryo)

Part B: Most of the purple color should be associated with the cell wall.

Part C: The LDH-Substrate solution should stain the cytoplasm light brown. The nuclei should not stain. The methylene blue will stain nuclei and the eosin will stain the cytoplasm.

IV Answers to Study Questions

1. Most of the peroxidase should be found in the cell wall which provides one of the many pieces of evidence that the enzyme acts on cell wall components.

2. All steps in anaerobic glycolysis occur in the cytoplasm.

3. Mitochondria - Incubate cells with a colorless electron acceptor that is converted to a colored product when it is reduced. Then examine the cells by microscopy to detect the colored product in the mitochondria.

Experiment 1 (701). Enzyme Cytochemistry

General Background

A. ENZYME CYTOCHEMISTRY

Most cells are colorless, translucent, and almost invisible in an ordinary light microscope. Consequently, biologists frequently stain cells to render them visible for microscopy. A number of organic dyes are available which can be used to stain biological specimens and some of these stains show a preference for particular parts of the cell. For example, the basic dyes methylene blue and toluidine blue stain cell nuclei, acid dyes like eosin stain the cytoplasm, sudan black stains fat droplets and janus green stains mitochondria. Although these dyes are useful for identifying cell organelles, they lack the specificity required to detect individual macromolecules.

A variety of new methods are now used by cell biologists for detecting specific macromolecules at the light microscopic level and you will use one of these methods in today's laboratory. The method is called enzyme cytochemistry, which permits the localization of specific enzymes in cells or tissues. Enzyme cytochemistry can be used to study the subcellular distribution of specific enzymes and can tell us if an enzyme is found in a particular organelle. In addition, the method is a powerful tool for identifying specific cell and tissue types.

The basic steps in enzyme cytochemistry are diagramed in Figure 1-1 and described below.

1. Fixation

   Cells or tissues are usually "fixed" by immersion in acids, organic solvents such as ethanol or acetone, or reactive aldehydes. Cells are fixed prior to most staining procedures in order to make them permeable to the stains. In addition, fixation causes cells to adhere to the microscope slide and cross-links cell proteins which locks or "fixes" them in position.

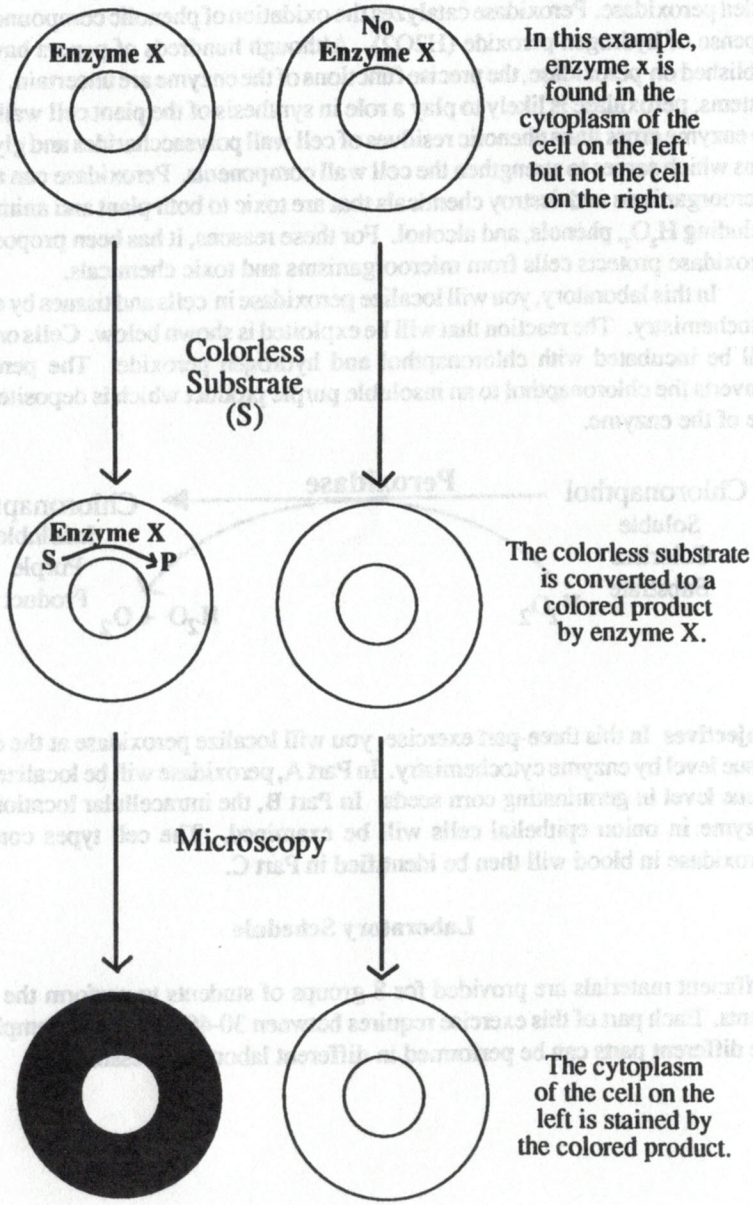
2. Incubation with a special colorless enzyme substrate

   Fixed cells or tissue sections are incubated with a colorless substrate of the enzyme under study and the enzyme catalyzes the conversion of the substrate to a colored product.

3. Detection of the colored product by microscopy

   The colored product deposited at the site of enzyme action is then detected by microscopy.

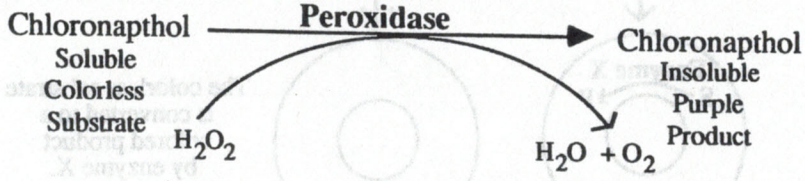
Figure 1-1. Enzyme Cytochemistry.



B. PEROXIDASE

The enzyme that you will study in Parts A and B of today's laboratory is called peroxidase. Peroxidase catalyzes the oxidation of phenolic compounds at the expense of hydrogen peroxide (H2O2). Although hundreds of papers have been published on peroxidase, the precise functions of the enzyme are uncertain. In plant systems, peroxidase is likely to play a role in synthesis of the plant cell wall. Here, the enzyme cross links phenolic residues of cell wall polysaccharides and glycoproteins which serves to strengthen the cell wall components. Peroxidase can also kill microorganisms and destroy chemicals that are toxic to both plant and animal cells including H_2O_2, phenols, and alcohol. For these reasons, it has been proposed that peroxidase protects cells from microorganisms and toxic chemicals.

In this laboratory, you will localize peroxidase in cells and tissues by enzyme cytochemistry. The reaction that will be exploited is shown below. Cells or tissues will be incubated with chloronapthol and hydrogen peroxide. The peroxidase converts the chloronapthol to an insoluble purple product which is deposited at the site of the enzyme.



Objectives In this three-part exercise, you will localize peroxidase at the cell and tissue level by enzyme cytochemistry. In Part A, peroxidase will be localized at the tissue level in germinating corn seeds. In Part B, the intracellular location of the enzyme in onion epithelial cells will be examined. The cell types containing peroxidase in blood will then be identified in Part C.

Laboratory Schedule

Sufficient materials are provided for 8 groups of students to perform the experiments. Each part of this exercise requires between 30-40 minutes to complete and the different parts can be performed in different laboratory sessions.

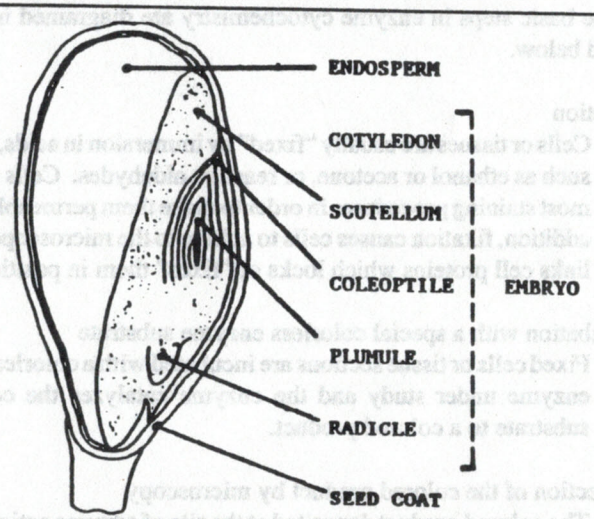
Part A. Peroxidase in the Germinating Corn Seed

Objective - Part A: To identify the sites of respiration, starch storage, and peroxidase in the germinating corn seed.

A. SEED GERMINATION

The seeds of flowering plants are typically resistant structures in which embryonic plants are enclosed. The outer layer of the seed is called the seed coat which protects the embryo from adverse conditions. The structures of the seeds of flowering plants are similar in that each seed contains a seed coat, an embryo, and a food storage tissue. The structures in a mature corn seed are shown in Figure 1-2. The mature corn embryo has a single cotyledon, or seed leaf, which is made up of a food-adsorbing portion called the scutellum and the coleoptile which forms a protective cap over the shoot. Below the cotyledon is the plumule, the apex of the embryonic shoot. The basal end of embryo, the radicle, develops into the primary root when the seed germinates. The food for the development of the corn seedling is largely located in the endosperm. In fact, the embryo is embedded in the cellular endosperm, the cells of which are rich in stored protein and especially starch. The endosperm is surrounded by several layers of cells that form the aleurone layer, in which a variety of enzymes are produced during seed germination.

Figure 1-2. Longitudinal Section of the Corn Seed.



Mature seeds have a low water content and the cells of the embryo are biochemically dormant. The cells can be activated in seeds by environmental factors, especially an increase in the moisture content of the atmosphere. This activation is called germination which begins by the uptake of water (imbibition phase) and culminates in the protrusion of the embryonic root from the seed. In corn, germination requires 24-48 hours under ideal conditions, and can be divided into the following steps:

1. Imbibition of water and hydration of subcellular organelles

2. Activation and new synthesis of a variety of enzymes in the aleurone layer and in the embryo

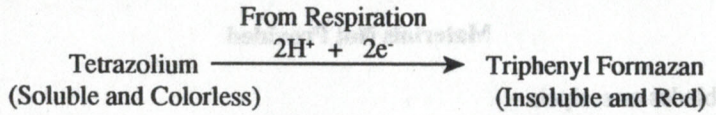
3. An increase in the levels of digestive enzymes amylase and proteinases in the aleurone layer and scutellum. These enzymes break down the stored starch and proteins into glucose and amino acids in the endosperm.

4. The transport of the breakdown products (glucose and amino acids) from the endosperm to the embryo where they are used as a nutrient source for the growing embryo

5. An increase in oxygen uptake and respiratory activity of the embryonic cells

6. Cell division and differentiation in the embryo and the emergence of the root from the seed.

Seed-testing laboratories frequently determine the percentage of viable (living) seeds in a seed lot by growing seeds under prescribed conditions and observing the number that germinate. The activation of respiration during germination forms the basis of a simple alternative test for seed viability. In this test, seeds are incubated with a colorless tetrazolium dye. Electrons produced by cellular respiration reduce the dye to a red product as shown in the equation below. Tetrazolium reduction has been used to localize respiring tissues in plants and animals and to detect mitochondria in viable cell preparations. In this exercise, you will use this histochemical procedure to identify the sites of respiration in the germinating corn seed.



Objective - Part A To identify the sites of respiration, starch storage, and peroxidase in the germinating corn seed.

Materials Provided

Germinating Corn Seeds (Note: the seeds must be soaked as described below 1-2 days before the laboratory session.)
*Tetrazolium (Freshly prepared)
*Potassium Iodide
*Peroxidase Substrate Solution (Freshly prepared) - This solution containing hydrogen peroxide, chloronapthol and Tris buffer should be made up 1-10 minutes before the experiment.
Transfer pipets

*Prepared as described in the instructor manual.

Materials Not Provided

Razor blades or scalpels
Hand lens or binocular low power microscopes (optional)
Distilled or deionized water
Forceps or toothpicks
A water incubator maintained at 37°C (optional)
32 small petri dishes (e.g. 3.5 x 1.0cm) or 32 small tubes (e.g. 1.5ml micro tubes).

Procedure

I. Preparation of Germinating Corn Seeds

Note: This procedure must be performed 1-2 days before the laboratory session. The procedure can be performed by the instructor, in which case about 50 seeds should be prepared, or by 8 groups of students who should prepare about 5-6 seeds each.

1. Place a layer of filter paper or paper towels in a petri dish and add about 5 ml of water to moisten the paper.

2. Add about 5-6 seeds and cover the dish. The seeds will be ready for analysis after 1-2 days in the dish.

II. Anatomy of Germinating Corn Seeds

Using a razor blade, cut a germinating corn seed in half, lengthwise from one flat side to the other. Note the seed coat, the position of the aleurone layer between the endosperm and the seed coat, the endosperm, and the small triangular section of the embryo at the pointed end. Study the section of the embryo with a hand lens or binocular microscope and attempt to identify the cotyledon, plumule, and radicle. (See Figure 1-2)

III. Location of starch, peroxidase, and the sites of respiration in seeds.

1. Obtain four small tubes or dishes and label them #1 - #4 with a marking pen.

2. Using a large transfer pipet, place 1ml of the following into the tubes. (Do not mouth pipet these solutions.) The iodine is a stain for starch, tetrazolium for respiration activity, and the peroxidase substrate for peroxidase.

Tube Number	
#1	Water
#2	Iodine
#3	Tetrazolium Solution
#4	Peroxidase Substrate Solution

3. Using a razor blade, cut 4 seeds in half as described above, and rinse the seed halves in water.

4. Place two halves in the solutions in each tube or dish such that the cut surfaces are in contact with the solutions. The seeds should be transferred with forceps or by piercing them with toothpicks.

5. After 3 minutes, remove the seeds from tube #2, rinse the seed halves in water, and note the seed areas that have stained with iodine and those that have not. Note - use forceps or toothpicks to transfer the seeds.

6. After 20-30 minutes, use forceps or toothpicks to remove the seeds from tube #1, 3, and 4. This time period can be reduced to 10 minutes if the incubation is carried out at 37°C. Rinse the seeds in water and note the colored seed areas in treatment groups 3 and 4. A hand lens or a binocular microscope can be used to study the colored areas of all seeds in more detail.

Data Analysis

In the space provided below, record the seed areas that are stained by each treatment.

Tube Number	Treatment	Result
1	H_2O	
2	Iodine	
3	Tetrazolium	
4	Peroxidase Substrate	

Part B. Intracellular Location of Peroxidase

Two approaches are frequently used to locate a specific enzyme within a cell. The first involves isolating cell fractions that contain different organelles by physical techniques such as centrifugation. The amount of the protein in each fraction is then determined by a biochemical procedure (e.g. an enzyme assay). For example, a mitochondrial fraction can be prepared from cells by differential centrifugation and the mitochondrial enzyme cytochrome oxidase should be found only in this cell fraction. The second approach consists of the direct detection of the enzyme in the cell by a specific staining procedure like enzyme cytochemistry. Each approach has advantages and limitations, and frequently both are used to definitely identify the subcellular site of an enzyme. Both approaches have been used to localize peroxidase to a specific site in plant cells, and you will now identify this site in onion epithelial cells by enzyme cytochemistry.

Objective-Part B To localize the site of peroxidase in onion epithelial cells by enzyme cytochemistry.

Materials Provided

Onion Bulb - The bulb should be cut into about 10 sections at the beginning of the experiment.
*Potassium Iodide (from Part A)
*Peroxidase Substrate Solution (Freshly prepared) - This solution containing hydrogen peroxide, chloronapthol and Tris buffer should be made up 1-10 minutes before the experiment.
Transfer Pipets

*Prepared as described in the instructor manual

Materials Not Provided

Razor blades or scalpels
Distilled or deionized water
Forceps
Microscopes
Microscope slides (Slides with frosted ends are preferred.)
Cover slips
Dissecting probes, straight pins, or needles
Ethyl alcohol

Procedure

1. A bulb is a short stem wrapped in fleshy modified leaves which are called bulb scales. The scales are covered with a single layer of epidermal cells which will

be used for the experiment. Remove one of the fleshy "scale" leaves from an onion bulb section and place it on the bench in front of you, concave surface up.

2. Obtain three clean glass slides and label them #1-#3 with a pencil.

3. With forceps, peel off three small pieces of the outer epidermal layer (~ 1cm x 2cm) and place one piece evenly onto each slide. If the tissue becomes badly wrinkled, discard it and obtain a new piece.

4. Place a few drops of ethyl alcohol onto the epidermis on each slide in order to fix the tissue. Add fresh alcohol to the tissue after 1 minute, and then let the slides air dry for a few minutes.

5. Place a few drops of the iodine solution onto the tissue on slide #1, water on slide #2, and Peroxidase Substrate on slide #3. Iodine staining is done to highlight internal structures of the cells.

6. After about 8 minutes, add a few additional drops of iodine onto slide #1, water onto slide #2, and Peroxidase Substrate onto slide #3.

7. After 10 minutes, place a paper towel at the edge of the tissue sections to draw off the solutions on the slides. Place a few drops of water onto each tissue section and draw off the water using a paper towel. Note the color of the tissue sections.

8. Place a few drops of water onto each section. Using a dissection probe or needle, slowly lower a cover slip over the tissue sections attempting to avoid trapping air bubbles.

9. Examine slide #1 with the low-power objective and then examine the cells under high power. Identify the cell walls that surround the individual cells, the cytoplasm, and finally the nuclei which should appear as brown staining bodies in the translucent cytoplasm. Next, examine slides #2 and #3, identify the above structures, and carefully note the subcellular distribution of the purple color in slide #3.

Data Analysis

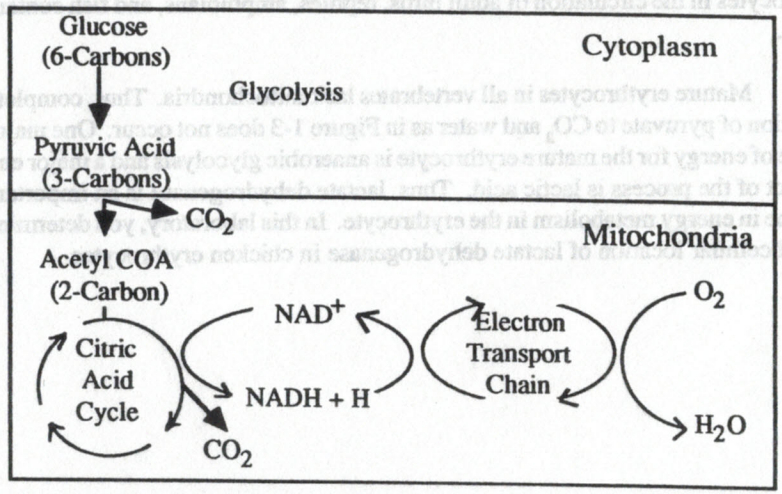
The student should provide a written description or a drawing of the subcellular distribution of peroxidase activity (purple color) on slide #3.

Part C. Intracellular Location of Lactate Dehydrogenase

A. METABOLISM AND LACTATE DEHYDROGENASE

The oxidation of glucose proceeds through many reactions, each catalyzed by a specific enzyme. Although the process appears complex, the basic patterns can be described in simple terms as outlined in Figure 1-3. Glucose is split into two molecules of pyruvic acid, each with three carbon atoms. These reactions occur in the cytoplasm by a process called glycolysis. In the presence of oxygen (aerobic metabolism) pyruvic acid enters the mitochondria, where it is converted to the acetyl group (2-carbon) of acetyl coenzyme A. The acetyl group is then completely degraded to CO_2 and water in the citric acid cycle and in the electron transport chain. During the citric acid cycle, the carbon atoms of the acetyl groups are liberated as CO_2, while, the hydrogen atoms (protons + electrons) are transferred primarily to the carrier molecule NAD^+ which is thereby reduced to $NADH+H^+$. The electrons are then transferred from the $NADH+H^+$ to a series of electron carrier molecules that comprise the electron-transport chain. During the course of this transfer, the electrons lose energy which is transferred to ATP. The terminal step in the chain is when the electrons and protons combine with molecular oxygen to form water.

Figure 1-3. Summary of Respiratory Metabolism.



NAD^+ = Nicotinamide-adenine dinucleotide

$NADH+H^+ = NAD^+ + 2$ electrons + 2 protons

Anaerobic metabolism occurs in the absence of oxygen. In the process, pyruvate is converted to 2-carbon or 3-carbon end products which then exit the cell. The process is frequently called fermentation or anaerobic glycolysis and the end products include ethanol, acetic acid, and lactic acid. In organisms such as brewers yeast and some bacteria, pyruvate is converted to ethanol and the process forms the basis of beer and wine production. Pyruvate can also be reduced to lactate in a reaction which is catalyzed by the enzyme lactate dehydrogenase (LDH). This reaction, which is shown below, occurs in most cell types. However, it is especially pronounced in lactic acid producing bacteria, in active muscle cells which frequently display oxygen deficiency during exercise and in a few cell types such as red blood cells, which lack mitochondria..

Lactate Dehydrogenase (LDH)

Pyruvate + NADH + H $\rightleftharpoons$ Lactic Acid + NAD

B. CELL TYPES IN BLOOD

Blood contains erythrocytes, leukocytes, and platelets suspended in a liquid medium called plasma. The erythrocytes, or red blood cells are the most numerous cell type in blood. These cells carry hemoglobin in the circulation and this protein transports oxygen from the lungs to tissues. Mammalian red cells are formed in the bone marrow of the adult by a process called erythropoiesis and then lose their nuclei shortly before they are released into the circulation. In contrast, erythropoiesis in nonmammalian vertebrates does not involve nuclear loss. Thus, erythrocytes in the circulation of adult birds, reptiles, amphibians, and fish contain nuclei.

Mature erythrocytes in all vertebrates lack mitochondria. Thus, complete oxidation of pyruvate to CO_2 and water as in Figure 1-3 does not occur. One major source of energy for the mature erythrocyte is anaerobic glycolysis and a major end product of the process is lactic acid. Thus, lactate dehydrogenase is an important enzyme in energy metabolism in the erythrocyte. In this laboratory, you determine the subcellular location of lactate dehydrogenase in chicken erythrocytes.

C. DETECTION OF LDH ACTIVITY

You will use an LDH substrate solution to detect the subcellular location of the enzyme. This solution contains chemicals that serve to couple the reaction catalyzed by lactate dehydrogenase to a color producing reaction as indicated in Figure 1-4. The highly colored brown product localizes in the area of the cell that contains LDH activity. You will also stain the erythrocytes with methylene blue which stains nuclei and eosin which stains the cytoplasm. These dyes will help you to identify these major regions of the erythrocytes.

Figure 1-4. Detection of LDH Activity

Lactate Dehydrogenase

1. Lactate + NAD $\longrightarrow$ Pyruvate + NADH

2. NADH + PMS $\longrightarrow$ NAD + PMS-H

3. PMS-H + TNBT $\longrightarrow$ PMS + TNBT-Formazan (brown product)

Abbreviations:

NAD - Nicotinamide adenine dinucleotide
NADH - Nicotinamide adenine dinucleotide, reduced
PMS - Phenazine methosulfate
TNBT - Tetranitrobue tetrazolium

Objective - Part C To identify the location of lactate dehydrogenase in chicken erythrocytes.

Materials Provided

8 Chicken blood smears
*LDH-Substrate - This solution should be made up immediately before use as described in the Instructor Guide.
Transfer pipets
Dishes for slide incubation
Methylene Blue
Eosin
*Nonidet P-40 (NP-40)

* Made up as described in the Instructor Guide.

Materials Not Provided

Ethyl alcohol (90-100%)
Distilled or deionized water
Microscopes, cover slips, and immersion oil
Small (100ml) beakers
Waterproof markers
Water bath maintained at 37°C (98°F)

Procedure

I. Fixation

1. Place the slide on the laboratory bench in front of you with the frosted side up. Place your initials on the frosted surface using a pencil. The cells have been spread on this surface of the slide. Place several drops of ethyl alcohol on the slide and after two minutes drain the slide by standing it on end.

2. Allow the slide to dry in air for a few minutes.

3. Rinse the slide with water. To rinse the slide, hold it on the frosted edge at a 90° angle over an empty beaker. Draw a few mls of water into a transfer pipet, slowly expel the water onto the top of the slide near the frosted edge, and permit the water to flow down the slide over the cells. Repeat this process three times.

4. Rinse the slide one time with about 3ml of the detergent NP-40 as described in Step 3. Do not apply the detergent directly onto the cells. The detergent will help to permeablize the cells which will facilitate the uptake of the LDH substrate.

5. Rinse the slides with water as described in Step 3.

II. Detection of LDH Isoenzymes

1. Preparing the LDH substrate solution: The instructor should prepare the LDH substrate solution. To prepare the solution, transfer the contents of the LDH-Substrate tube to 50ml of distilled water and stir for a few minutes until the powder is dissolved. The solution should be protected from light as much as possible and used immediately after preparation.

2. Place 15ml of the substrate solution into 3 of the plastic dishes. Do not mouth pipet this solution.

3. Place the 8 slides into the three dishes, frosted side up.

4. Place the lids on the dishes and then float the dishes in a water bath at 37°C for 30 minutes.

NOTE: Great care should be taken not to bump the dishes during the incubation.

5. Rinse the slide with water as described in Section I-3 and then drain the slide by standing it on end for about 5 minutes.

III. Staining with Methylene Blue and Eosin

1. Place the dry slide on the laboratory bench in front of you with the frosted side up and place a ruler next to the slide.

2. Pipet 5µl of methylene blue onto the slide about 1 cm away from the end of the slide that is opposite to the frosted end.

3. Pipet 5µl of eosin onto the slide about 2 cm away from the end of the slide that is opposite to the frosted end.

4. After about four minutes, rinse the slide with water as described in Section I-3 and air dry for about five minutes.

IV. Microscopy

1. Examine the sections of the slide that were stained with methylene blue and eosin with the low power objective of your microscope and identify the nucleus and cytoplasm of the erythrocytes. Examine several sections of the slide that were not stained by these dyes in order to determine the location of the brown product which is indicative of LDH activity.

2. Examine the three regions under oil immersion (if available) with a cover slip or under the highest power objective of your microscope to observe the erythrocytes in more detail. Record the regions of the cells that have stained with methylene blue, eosin and the LDH-substrate.

Study Questions
(For Parts A, B, and C)

1. It has been proposed that peroxidase in plants catalyzes a reaction which serves to strengthen the cell wall. Does the subcellular distribution of peroxidase in onion cells provide support for this view? Explain.

2. What subcellular region of chicken erythrocytes contains the highest concentration of LDH activity? Does this localization of LDH agree with what is known about metabolic processes that occur at this site?

3. Artificial electron acceptors can be used to detect a specific organelle by cytochemistry. Name this organelle and outline a procedure that you would use to detect this organelle by cytochemistry.

Selected References

Seed Physiology, Volume II. "Germination and Reserve Mobilization", D.R. Murray (ed.). Academic Press, 1984.

Molecular and Physiological Aspects of Plant Peroxidases, H. Greppin, C. Pend, and T. Gaspar (ed.) University of Geneva, Switzerland, 1986.