# Exercise 15-C
# PHYSIOLOGICAL CHARACTERISTICS OF BACTERIA CONTINUED: GELATIN LIQUEFACTION, TSI, SIM, UREA HYDROLYSIS AND NITRATE REDUCTION

## Utilization of Proteins, Amino Acids, and Other Nitrogen Containing Compounds

Many bacteria can degrade a variety of proteins and utilize the resulting peptides and amino acids to synthesize their own cellular components. They can also catabolize amino acids as a means of providing some of the energy (ATP) needed for cellular function. Microorganisms vary from species to species with regard to their **proteolytic ability**, so these features can be useful when characterizing different organisms.

## Gelatin Hydrolysis or Gelatin Liquefaction

The protein **gelatin** is obtained through the hydrolysis of **collagen**, a component of animal connective tissue. Since many bacteria produce **gelatinase enzymes** that catalyze the hydrolysis of gelatin, this protein is a convenient substrate to use when testing for proteolytic enzyme activity in microorganisms.

Nutrient gelatin appears to be a liquid or broth medium when maintained at room or incubator temperatures, but will solidify when placed in an ice bath (or refrigerator) for a period of time (much like jello). When inoculated with microbial cultures and allowed to incubate for a period of time (2-14 days), tubes of nutrient gelatin can readily show protein catabolism. If the gelatin is catabolized by the action of microbial enzymes, the medium will remain liquid when cooled (gelatin liquefaction has occurred and hydrolysis is positive). If the medium is solid when cooled, gelatin liquefaction has not occurred and hydrolysis is negative.

**Procedure:**

1. Inoculate one tube of nutrient gelatin by stabbing a sample of the culture being tested to the tube bottom. Incubate the tube at an optimum growth temperature ($37^{o}$ C or room temperature depending on culture type), for 2 to 14 days. Gelatin hydrolysis may proceed very slowly.

2. To examine for hydrolysis, chill the tube in an ice water bath using a sterile tube of nutrient gelatin as a control. The control tube and the tubes in which no hydrolysis has taken place will solidify, while the hydrolyzed gelatin will remain liquid.

   **Note** – If gelatin liquefaction is proceeding slowly from the surface downward, as it often does, agitation can mix hydrolyzed with unhydrolyzed gelatin creating a combination that will solidify giving a false negative result. **DO NOT SHAKE TUBES!**

## Amino Acid Degradation to Yield Hydrogen Sulfide and Indole

The activity of some bacteria on sulfur-containing amino acids, e.g., cysteine and methionine, results in the liberation of **hydrogen sulfide** ($H_2S$) gas. Common examples of this reaction are found in the "aroma" of rotting eggs and in the blackening of certain spoiled canned foods. In the later instance, the blackening results from a reaction between the $H_2S$ formed by the bacteria and the metal of the can.

Production of $H_2S$ by bacteria cultures can be demonstrated in the laboratory if sulfide-producing cultures are grown in media containing salts of metals such as bismuth and iron. The appearance of a dark color in the medium (toward the tube bottom) is due to the formation of metal sulfides (typically **iron sulfide**), a black precipitate.

**Indole** is an aromatic, nitrogen-containing organic compound formed from the degradation of the amino acid **tryptophan** by various bacteria. Indole formation involves the catalytic activity of proteins called **tryptophanase** enzymes or tryptophan indole-lyase enzymes. The importance of the indole test lies in the fact that only certain bacteria form indole, and it can be readily detected chemically. Thus the degradation of tryptophan is useful as another differentiating physiological reaction.

**Triple Sugar Iron Agar (TSI)**

Triple sugar iron agar (TSI) is a differential medium that indicates the ability of organisms to ferment lactose, sucrose, and glucose, with the formation of acid and gas, and also their ability to produce hydrogen sulfide ($H_2S$) within a single tube.

Inoculation of the medium with organisms able to ferment one or more of the carbohydrates present and produce acid will cause the **phenol red** indicator in the medium to change from red to yellow. This color change may be visible throughout the slant or may be restricted to a certain area. In general, the results for glucose and sucrose fermentation are read in the tube butt (bottom) while the results for lactose fermentation are read in the slant (upper portion). Inoculation of TSI with *Klebsiella pneumoniae* or *K. oxytoca* (capable of fermenting all three sugars) will usually result in a color change from red to yellow throughout the medium, while inoculation with *Serratia marcescens* results in the production of acid and sometimes gas from the fermentation of sucrose and glucose, but not from lactose. This is indicated by a color change in the butt of the tube, but no change in the color of the slant. Organisms such as *Enterobacter aerogenes* and *E. cloacae* that can ferment glucose but form acetoin (a neutral end product) will often leave the slant portion of the TSI medium red (a **false negative for lactose fermentation)**. *Proteus vulgaris* cannot ferment lactose, but will form enough acid through the fermentation of glucose and sucrose to turn the entire medium yellow (a **false positive** for lactose fermentation). Since the fermentation of glucose and sucrose are indicated together in the tube butt, and false readings for lactose fermentation are fairly common, TSI medium provides a less reliable indication of carbohydrate fermentation than do individual carbohydrate deeps.

The **TSI** medium also indicates the ability of organisms to degrade sulfur-containing amino acid molecules and produce **hydrogen sulfide**. The sulfur released as $H_2S$will bind with iron in the medium to form a **black precipitate** called **iron sulfide** (FeS). In some cases, $H_2S$ production is indicated by the formation of a small amount of black precipitate (iron sulfide) between the butt and the slant, in others the entire tube bottom will turn black. If iron sulfide turns the entire lower portion of the tube black, it will not be possible to read the results for sucrose and glucose fermentation.

**Procedure:**

1. Obtain one tube of TSI agar and inoculate it with your unknown culture by making a zigzag streak up the slant (bottom to top) and stabbing the culture to the tube bottom. Carefully label and incubate this tube at $37^o$ C until the next laboratory period.

2. Examine the tube contents and record the data and results obtained. Compare your results with those obtained by other members of the class.

**Sulfur Indole Motility (SIM) test medium**

SIM is a differential medium that can be used to demonstrate hydrogen sulfide production and indole production in a single tube. The medium also contains material that allows for the determination of motility. Non-motile organisms will grow only along the line of inoculation (the line will be distinct and easily visible), while motile forms will show diffuse growth or turbidity throughout the medium.

**Procedure:**

1. Obtain one tube of SIM medium and inoculate it with your unknown culture by making a single stab to the tube bottom. **Note** – Use a loop with a long, straight wire, and try to stab down and out along the same line. Label the tube and incubate it at $37^{o}$ C until the next laboratory period.

2. Observe the tube for motility and $H_2S$ production (turbidity or black precipitate throughout the medium), then test for the presence of indole using the method of Kovac. Add a thin layer of Kovac's reagent to the surface of the SIM medium (enough to be clearly visible). The appearance of a **deep red** color **in the Kovac's reagent** within a few minutes indicates that indole is present. If the Kovac's reagent remains yellow, indole is not present. Compare your results with those obtained by other members of the class.

3. Record your data and results. **Note** – If SIM medium is allowed to sit for an extended period of time after applying Kovac's reagent, a reddish color will appear **within** the agar medium. This should **not** be recorded as a positive test.

**Urea Hydrolysis**

The hydrolysis of urea by *Proteus* (and multiple other species) results in the production of ammonium due to the action of the enzyme **urease**. This alkaline reaction can be demonstrated in urea agar containing the pH indicator **phenol red**. A positive urease reaction (hydrolysis of the urea) is indicated by a change in color from yellowish-peach (pH 6.8) to a red-cerise (hot pink) color (pH 8.1 or more alkaline).

**Procedure:**

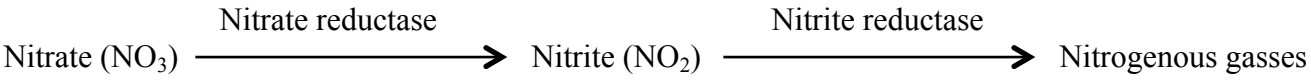
1. Obtain one slant of urea agar and inoculate it with your unknown culture by spreading cells from an agar plate over the entire slant surface (**do not** stab into the butt of the tube). Carefully label and incubate this tube at $37^{o}$ C for at least 48 hours.

2. Examine your tube for the action of urease, and record your data and results. Compare your results with those obtained by other members of the class.

**Nitrate Reduction**

Various types of bacteria are capable of using **nitrate**, instead of **molecular oxygen** ($O_2$) as a **final electron acceptor** at the end of their electron transport chain. Even *Pseudomonas aeruginosa*, an organism type usually described as being obligately aerobic, has this ability. Organisms with this capability can carry out **anaerobic respiration**, i.e., are able to use a respiratory or oxidative type of metabolism under anaerobic conditions.

The nitrate reduction test can be used to determine if or not bacteria can produce two different enzymes potentially involved in the reduction of nitrate, **nitrate reductase** and **nitrite reductase**. The reactions involved can be summarized as follows:




Some bacteria can produce both of these enzymes, some can produce nitrate reductase only and some can produce neither. The Nitrate Reduction Test uses the presence or absence of **nitrite** (after incubation) as an indicator of nitrate reduction. Determining the results is complicated by the possibility that nitrite can be reduced to Nitric oxide (NO), and this reduced further to yield additional nitrogenous gasses including nitrous oxide ($N_2O$), ammonia ($NH_3$), or free nitrogen gas ($N_2$), all of which will exit the medium. An inverted Durham tube within the medium helps to alleviate this problem by catching gasses (bubbles within the Durham tube would indicate production of nitrogenous gasses), but nitrate reduction tests yielding negative results for nitrite must still be subjected to an additional test.

If a culture is able to reduce nitrate to nitrite, the addition of sulfanilic acid (Nitrate reagent A) will result in the formation of a colorless complex (nitrite-sulfanilic acid). The addition of alpha-naphthylamine (nitrate reagent B) will then yield a red precipitate (prontosil). The formation of the **red color** shows **nitrite is present**, and should be recorded as a positive test for nitrate reduction (nitrate-positive); however, a lack of red color is not necessarily indicative of a negative result. If addition of the nitrate reagents does not yield a red color, nitrite is not present, but there are two possible explanations for this:

1) The organisms present in the medium do not make nitrate reductase, nitrate was not reduced and the culture is recorded as being nitrate-negative.
2) The organisms present produced both nitrate reductase and nitrite reductase, and the nitrogenous gasses formed have left the medium. Under these circumstances, the culture is recorded as nitrate-positive.

When organisms in nitrate medium yield a negative result, carful observation of the Durham tube and an additional step must be completed to determine which of the two explanations is accurate. Zinc particles can be added to nitrate medium, and will catalyze the reduction of nitrate to nitrite chemically. If the addition of zinc causes the medium to turn red, nitrate was not reduced by the culture, and the result is truly nitrate-negative. If the addition of zinc causes no color change, then both nitrate and nitrite were reduced, nitrogenous gasses were formed and the culture is nitrate-positive. Durham tubes within nitrate-positive cultures will likely contain gas bubbles (nitrogenous gasses).

**Procedure**

1. Obtain and inoculate one tube of nitrate agar by stabbing a loopful of culture to the tube bottom. Incubate the tube for 5-7 days at whatever temperature is most appropriate for your culture. If during inoculation you introduce bubbles into the medium, record their presence and appearance.

2. After incubation, observe the medium carefully to determine if or not the culture has grown, and record the presence or absence of gas bubbles inside the Durham tube.

3. Add five drops of Nitrate reagent A and five drops of Nitrate reagent B, and watch for the formation of a **red precipitate** on the medium surface (this will occur within a few minutes). Record this data, and record the culture as **nitrate-positive** (nitrate reductase was formed). **Note** – The formation of a thin pinkish layer is most likely due to the medium absorbing nitrogenous gasses from the atmosphere, and is **not** indicative of a positive result.

4. If after the addition of Nitrate reagents A and B there is no color change (or only a thin, pink layer), wet a sterile loop and use it to transfer a small amount of zinc powder from the container provided into the medium (stab the zinc at least 1cm into the medium).

5. Observe the tube after 5-10 minutes. If **no color change** occurs, nitrite is not present but the culture should be recorded as **nitrate-positive** because both nitrate reductase and nitrite reductase were formed resulting in gaseous end products that have left the medium. Gas bubbles should be present in the Durham tube. If a red precipitate forms along the stab line (zinc catalyzes nitrite formation) the culture is **truly nitrate-negative** and neither enzyme was formed.

6. Record your data, results and conclusions. If your culture is positive for nitrate reduction, be sure to specify the type of enzymes present, i.e., did the culture produce just nitrate reductase, or did the culture produce both nitrate reductase and nitrite reductase. Indicate the type of product formed from nitrate in your conclusion.

**Questions:**

1. Hydrogen sulfide ($H_2S$) is produced by certain bacteria that are able to catabolize what type of organic compounds?

2. The presence of hydrogen sulfide is indicated in TSI and SIM media by the formation of what?

3. Indole is produced when the amino acid _____ is degraded by certain bacteria. What reagent is used to test for the presence of indole?

4. How can you determine if or not organisms are motile by observing their growth pattern in SIM media?

5. What pH indicator is present in urea agar? What chemical change is responsible for the color change apparent in this medium when urea is catabolized.

6. What do some bacteria use nitrate for?

7. What two enzymes can be tested for with the Nitrate reduction test? What types of end products are formed by the catalytic activity of these enzymes?

8. What is the effect of zinc on nitrate?

**STUDY GUIDE**
**Exercise 15C**
**GELATIN LIQUEFACTION, TSI, SIM,**
**UREA HYDROLYSIS & NITRATE REDUCTION**

**Materials, Methods & Data:**

**GELATIN LIQUEFACTION**

Name of enzyme being tested for? _____ What reaction is catalyzed by this enzyme? Gelatin acted upon by the enzyme undergoes _____

An inoculated tube of nutrient gelatin medium must be subjected to what treatment (following several days of incubation) in order to read data and record results? _____

What does a positive gelatin liquefaction test look like? _____

What does a negative gelatin liquefaction test look like? _____

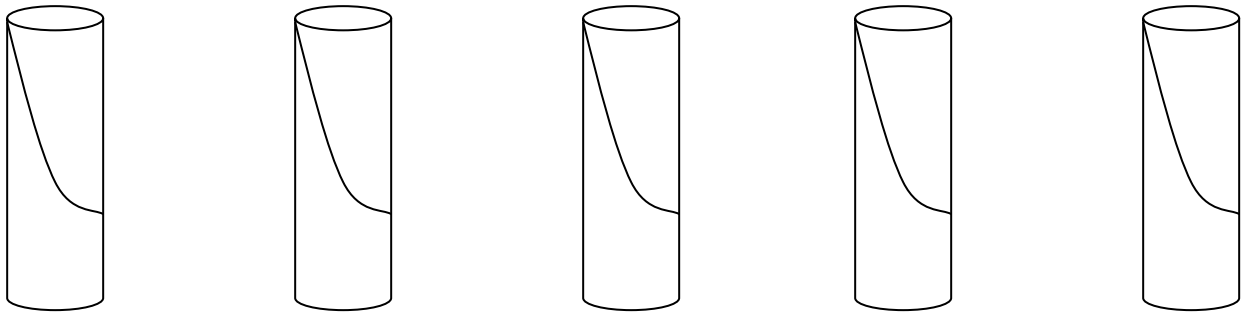
**TSI & SIM** **Date** _____

Sugars present in TSI _____

pH indicator _____ Neutral = ☐ Acidic = ☐ Alkaline = ☐

Black precipitate formed _____ What type of substance is being catabolized to form the precipitate? _____

TSI Data & Results

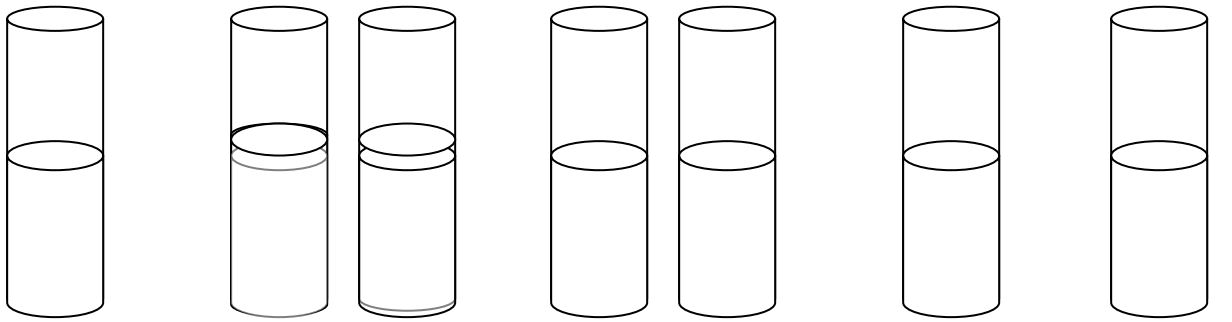


Initial color | $H_2S$ (+), Lac (–) | $H_2S$ (+), Lac (+) | $H_2S$ (–), Sug (+) | No reaction

SIM medium is used to test for _____
_____

Reagent used to detect indole _____ Motility is indicated by?
_____

**SIM Data & Results**

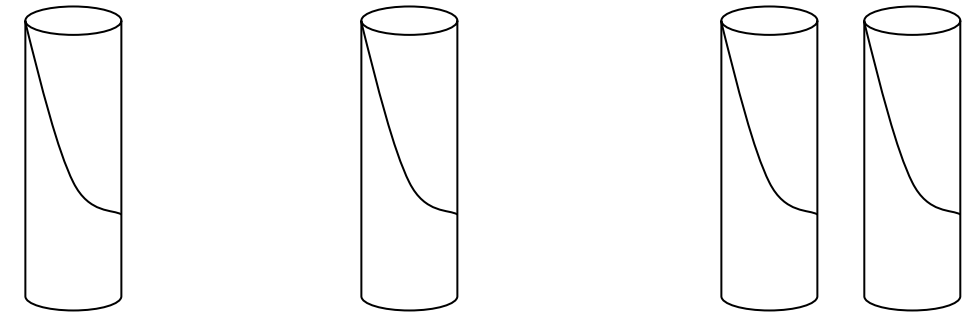


Initial color  Indole (+)  Indole (–)  $H_2S$ (+)  $H_2S$ (–)  motility (+)  motility (–)

**Urea hydrolysis**  **Date** _____

Enzyme tested for _____ Product formed _____

pH indicator _____ Neutral = ☐ Acidic = ☐ Alkaline = ☐



Initial color  Positive result  Negative results

**Nitrate Reduction**

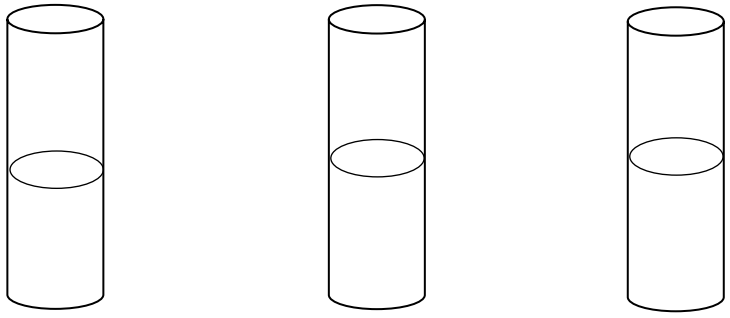
Enzymes being tested for are _____ and _____

What product is formed through the catalytic activity of the first enzyme, and how is it detected?

_____

What types of products are formed by cultures producing both enzymes, and what happens to them?

_____



A + B Positive  Zinc Negative  Zinc Positive