BIOLOGICAL MOLECULES

Pre-lab Preparatory Information

Know this material prior to attending your lab. You may be quizzed on this material.

Objectives: by the end of this lab, you will be able to…

…use a B & L Spectronic 20 spectrophotometer (Spec 20) to quantify biological molecules in a sample.

…interpret and explain the results of assays conducted in a urinalysis and use them to justify a diagnosis.

…describe and explain the molecular interactions that occur in the assays; in particular, what component of the molecule reacts with the specific reagent.

1. Urinalysis:

Urinalysis is a suite of analyses used to screen for diseases ranging from leukemia and diabetes to kidney disease and urinary tract infections. Analyses include simple macroscopic (naked eye) examinations of color and turbidity (cloudiness), microscopic examination of cell numbers, types, and shape (recall crenation from Lab 1) and chemical assays to quantify macromolecules. As in a real laboratory, your urinalysis will require the use of tools introduced previously, the **Spec 20, graphing,** and **assays**. For safety purposes, rather than real urine, you will analyze "artificial urine." Our assays focus on disorders associated with proteins and carbohydrates. Although other biological molecules have been omitted because of time limitations, assays can quantify lipids, nucleic acids, and many other compounds.

To detect most diseases, quantification of a specific substance is necessary to determine whether its concentration is higher (or lower) than normal; hence, the use of the assay. Examples of diseased or periodic conditions assayed in clinical laboratories include (Shmaefsky, 1990).

1. **Urine pH imbalances**: Usually, urine pH is between 5 and 7. Consistent acidic urine is a sign of metabolic or respiratory acidosis, methanol poisoning, or metabolic disorders (for example, phenylketonuria). Consistent alkaline urine is indicative of metabolic and respiratory alkalosis and urinary tract infections.

2. **Glycosuria**: (*glyco-* meaning sweet, *-uria* referring to urine) Urine normally has up to 0.6 mg glucose/milliliter. Higher levels of glucose are often associated with diabetes mellitus, but may also be due to pregnancy, excessive stress, renal tubular or brain damage.

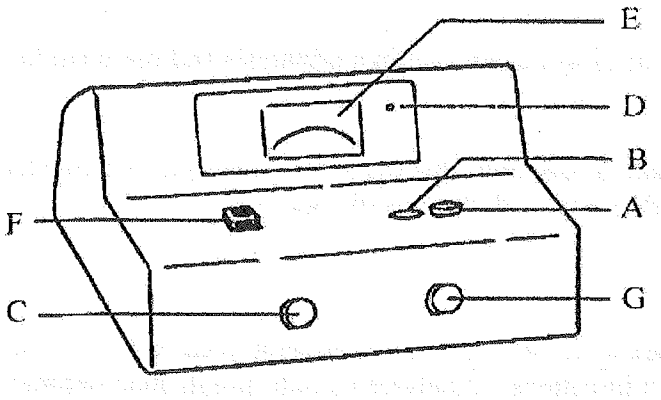
3. **Proteinuria**: Normal urine contains up to 0.3 mg of albumin per milliliter. A high level of protein in the urine is an indicator of glomerular damage in the kidney. It may also result from excessive exercise, cold exposure, and acute abdominal disease.

In-Laboratory Exercises:

Work in Groups of 4

Exercise 1: Use of the B & L SPECTRONIC 20 SPECTROPHOTOMETER.

This exercise reviews the use of the Spec 20 so that you can use this tool effectively when you conduct your urinalysis. The major components of an analog Spec 20 are illustrated below:



A. Wavelength control
B. Wavelength selection (in nanometers)
C. Dark Control
D. On/off indicator jewel
E. Transmittance and absorption meter (Only use absorption!)
F. Sample holder
G. Light control

a. Color and Wavelengths: To appreciate how the Spec 20 works and to confirm the relationship between the visible colors and wavelengths, do this simple exercise before reviewing how to work with a real sample.

i. Place a small piece of chalk (ca. 1 cm long) into the bottom of a dry Spec 20 tube and insert the tube into the Spec 20.

ii. Look down into the tube while you rotate the wavelength knob. This works best with most of the room lights off since the light intensity is low.

iii. Record the observed color that corresponds to each wavelength.

Do these colors agree with those given in your textbook?

Table 1

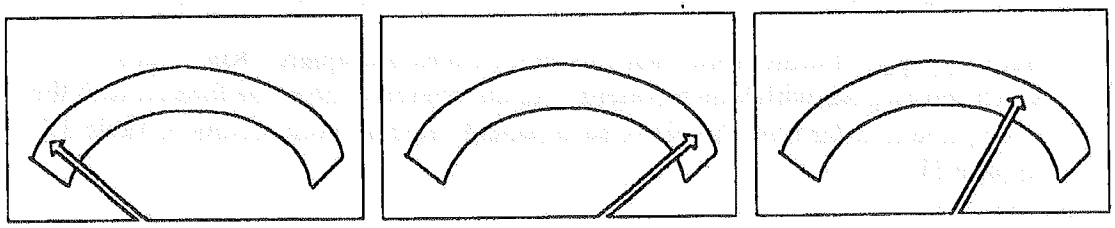
Wavelength (nm)	Color Observed
400	
425	
450	
475	
500	
525	
550	
575	
600	
626	
650	
675	
700	

b. Standard Operating Procedure: The basic procedures for operating an **analog "Spec 20"** are detailed below. Analog Specs have a dial with a pointer rather than a digital readout. Go through these steps and learn them BEFORE you attempt any assay. Knowledge of your tool provides better data in a shorter amount of time. Be sure to know how to use this tool!

1. Rotate the wavelength control (A) until the desired wavelength (in nanometers) is indicated by the wavelength scale (B).
2. Turn the instrument on by rotating the dark control (C) clockwise. When the instrument is on, the jewel (D) will glow.
3. Allow at least 10 minutes warm-up time. Bring the meter needle to "∞" (infinity) on the absorbance scale (lower, E) by adjusting with the dark control (C).
4. Insert a spectrophotometer tube containing the BLANK into the sample holder compartment (F), making certain that the tube is firmly lodged and that the etched mark on the tube faces you. Close the lid gently.
5. Rotate the light control (G) until the meter (E) indicates zero on the absorbance scale. This procedure calibrates the instrument relative to the blank.
6. Once calibrated, the sample may be inserted in place of the blank and the absorbance read directly from the meter.

   Note that this is a logarithmic scale so that the value of each mark changes as the absorbance increases. For example, each mark between 0.1 and 0.2 is worth 0.01 units, while the mark between 0.8 and 0.9 is 0.85 (not 0.81).
7. For a series of samples in the same solvent, a single blank calibration usually is sufficient. However, it is desirable to check the calibration periodically when the instrument is to be used by several people over a period of time.

Perform the following after the machine is warmed up and the wavelength selected.

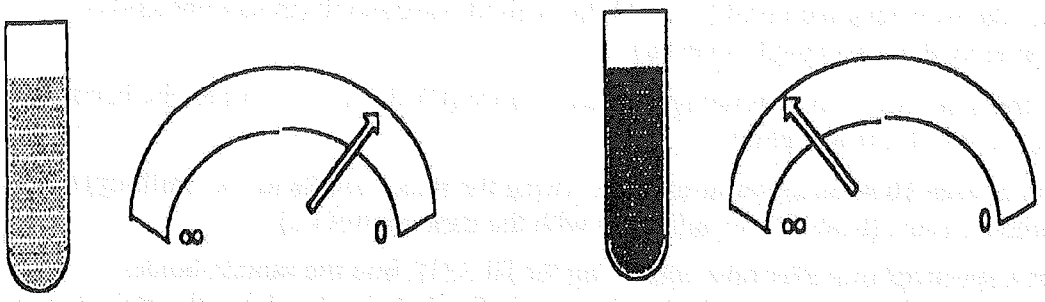


1. Needle when the dark current set to infinity (∞).
2. Needle at zero absorbance for the blank.
3. Reading for sample.

Summary

1. With NO sample in the sample holder, set the left side of the scale to infinite absorbance with the left knob (C, or "dark control" knob).
2. With the BLANK in the sample holder, set the right side of the scale to zero absorbance with the right knob (G, or "light control" knob).
3. Read each sample (standard and unknowns) directly off the absorbance scale.

Remember that absorbance is proportional to concentration. If the sample does not contain many molecules that absorb light, there will be little color and the absorbance will be low (left). If the sample contains more molecules that absorb light, there will be more color and the absorbance will be higher (right).



NOTE: Instructions for operating the digital Spec 20's are taped to the lab bench. **All practical exams will use the analog Spec 20's exclusively, so learn how to use them NOW.**

Exercise 2. Urinalysis Assays

I. Assay for pH Imbalances:

1. Obtain 9 squares of pHYDRION paper (a mixture of pH indicators). Place them on a paper towel.

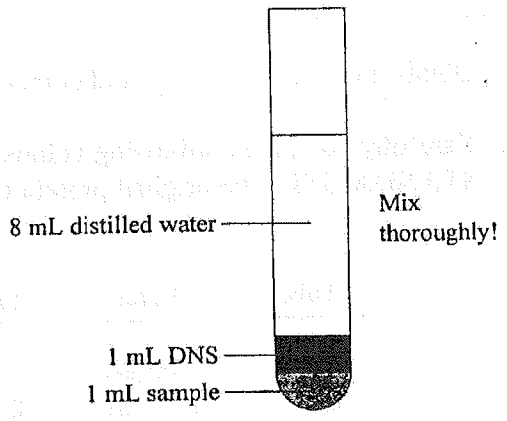
2. Place one drop of buffer, pH 7.0, on one square. Compare the color formed with the color guide provided. Repeat with different squares for pH 4.0 and 10.0.

3. Place one drop of urine from each patient on a separate square. Keep track of which square goes with which patient! Again, compare the color formed with the color guide to determine the pH of each patient. Record your results in Table 3.1 on page 11.

II. Assay for Glycosuria:

SAFETY NOTE: You must wear your chemical splash-proof safety goggles when adding DNS to each test tube (step 3) and when adding water and mixing (step 5). Students who forget to bring their goggles may watch, but at a safe distance.

1. Obtain 8 clean test tubes. Label them B, S, 1, 2, 3, 4, 5, and 6.
2. Add 1 mL of water to tube B (BLANK). Add 1 mL of glucose to tube S (STANDARD). Finally, add 1 mL of each patient's urine to the appropriately labeled tube making certain that you do not get the samples mixed up.
3. Add 1 mL of DNS to EACH tube (all eight) and mix thoroughly.
4. Place all tubes into the boiling water bath. After exactly 5 minutes, remove the tubes and cool them in running tap water.
5. Now add 8 mL (8 cc) of distilled water to each tube (all eight). Place a small square of Parafilm securely over the open end, then mix thoroughly.
6. Determine the absorbance (A) at 540 nm for each tube. Record your results in Table 3.2 on page 11.
7. The concentration of glucose in a sample is directly proportional to its absorbance. In the box, write an algebraic formula that expresses this relationship mathematically, using the following notation



C_u = concentration in unknown
C_s = concentration in standard
A_u = absorbance for unknown
A_s = absorbance for standard

Then rearrange the terms so you can solve for the concentration of the unknown. The standard (S) had glucose concentration of 2.0 mg per milliliter.

C_u =

Calculate the glucose concentration for each patient and record the results in Table 3.2. Show your work for one of these as "Sample calculation" in that table.

What happens to the units used to express concentration when you do this calculation? What units are used to express the concentration of the unknown?

Did any of the patients have higher than normal concentrations of glucose in their urine? How would you diagnose these patients?

What was the nature of the reaction between glucose and DNS? (Refer to the Reactions section.) What other substances might be in urine that would give a positive reaction with DNS? (Look up phenylketonurea in your textbook.)

III. Assay for Proteinuria:

[In this section you will also confirm the relationship between absorbance and sample concentration.]

SAFETY NOTE: You must wear your chemical splash-proof safety goggles when adding biuret reagent to each tube. Students who forget to bring their goggles may watch, but at a safe distance.

1. Obtain 11 clean test tubes. Label them as indicated on Table 3.3 on page 12.

2. Carefully pipette the following volumes into the first five tubes; these will be your STANDARDS. The original protein concentration is 4.0 mg/mL.

Tube	Water	Protein	Protein - final concentration
B	1.00 mL	0 mL	0 mg mL
S-1	0.75 mL	0.25 mL	1.0 mg/mL
S-2	0.50 mL	0.50 mL	2.0 mg/mL
S-3	0.25 mL	0.75 mL	3.0 mg/mL
S-4	0 mL	1.00 mL	4.0 mg/mL

3. In tubes P-1 through P-6, pipette 1.0 mL of each patient's urine, as indicated in Table 3.3. REMEMBER, do not get them mixed up!

4. Add 4.0 mL (4 cc) of biuret reagent to EACH tube (all eleven). Cover tubes with parafilm, mix, and incubate at room temperature for at least 30 min. [As before, ALL tubes contain the reagent, including the BLANK (B).]

5. Determine the absorbance (A) at 540 nm for each sample, recording your results in Table 3.3 (end of this lab).

6. On the bottom of Table 3.3, use the data from the standard (tubes B through S-4) to make a graph plotting the absorbance (vertical axis) for each different amount of protein (horizontal). The points should make a straight line.

7. Estimate the concentration in each patient's urine, using both the graph and an

equation comparable to that used for sugars (the absorbance for 4 mg/mL is most accurate). Fill in the results in Table 3.3.

Did any of the patients have more protein in their urine than normal? How would you diagnose these patients?

What part of a protein molecule reacts with the biuret reagent? Are there any other biological molecules that might react with the biuret reagent?

IV. Assay for Hemoglobinuria:

Since hemoglobin is a brownish-red, the presence of this compound can be determined by simply looking at each sample.

Presence of hemoglobin in urine (*).

Patient	1	2	3	4	5	6
Hemoglobin:						

What colors are absorbed by hemoglobin?

What wavelengths correspond to these colors? (Look up "electromagnetic spectrum" in your textbook. Don't forget to compare to your notes from Exercise 1.)

What wavelength should you use to determine the amount of hemoglobin in each sample?

What should you use as a standard?

Did any of the patients have excessive hemoglobin in their urine? How would you diagnose this problem?

V. Make and Defend Your Diagnoses:

Your lab director (in other words, your boss) asks for the results of your assays because she needs to send a report to the physician. As you prepare your report, keep in mind that **symptoms** are observations that indicate a possible disease (e.g., too much sugar in the urine). A name is often given to the set of symptoms (e.g., glycosurea). The **diagnosis** is that disease may cause this set of symptoms (e.g., diabetes mellitus).

What step of the Scientific Method would a diagnosis correspond to?
Compare the logical process that a physician uses to treat a patient with the Scientific Method.

Keep in mind that your boss does not want a lawsuit from a patient who was misdiagnosed, so be prepared to justify and defend your conclusions. If you get any wrong, heads will roll!

1. Did any of the patients' urine contain excess sugar? Justify and defend your conclusions. What is a likely diagnosis?

2. Did any of the patients' urine contain more protein than normal? Justify and defend your conclusions. What is a likely diagnosis?

Knowing that if a case is taken to court, methodologies are scrutinized, thus your lab director wants to make sure you did and can your analyses correctly and wants you to…

1. …review such basic terms as blank, standard, and unknown and explain how one uses the absorbance of the standard to calculate the concentration of material in the unknown sample.

2. …your results with the protein standard curve to explain how absorbance is proportional to concentration and explain how you used this to determine the amount of material in an unknown?

3. …report on whether or not any of the patients' urine appeared to have a pH imbalance, explain what pH is, and explain the difference between urine samples with a pH of 6 and a pH of 3.

4. …report on whether or not any of the patient's urine appeared to contain hemoglobin, how you would quantify the amount of hemoglobin in a urine sample. What would be in the blank? The standard? What wavelength would you use?

Reference

[Background information for this exercise can be found in the following publication; it is **not** a required reading.]

Shmaefsky, Brian R. 1990. Artificial urine for laboratory testing. *American Biology Teacher*. 52: 170-172.

COMING ATTRACTIONS:

This week you used a spectrophotometer to determine the amounts of sugar and protein in unknown samples. In the next lab you will use this instrument even more carefully to measure the increase in the amount of a substance. The RATE of increase will be used to quantify enzyme activity.

Name _____; Section _____; Date _____

Table 3.1. pH values for urine from different patients.

Patient:	1	2	3	4	5	6
pH:	7	7	6	7	8	5

Table 3.2. Glucose content for each patient's urine.

Patient:	1	2	3	4	5	6
Absorbance:						
Glucose (mg/mL):		.19	2.0			

Absorbance of 2.0 mg/mL glucose (S):

Sample calculation:

$5)\ \frac{x}{.5} = \frac{2}{.25}$ $\quad 1)\ \frac{x}{.1} = \frac{2}{.125}$ $\quad 1)\ \frac{x}{2} = \frac{2}{.125}$

$1.25x = 1$

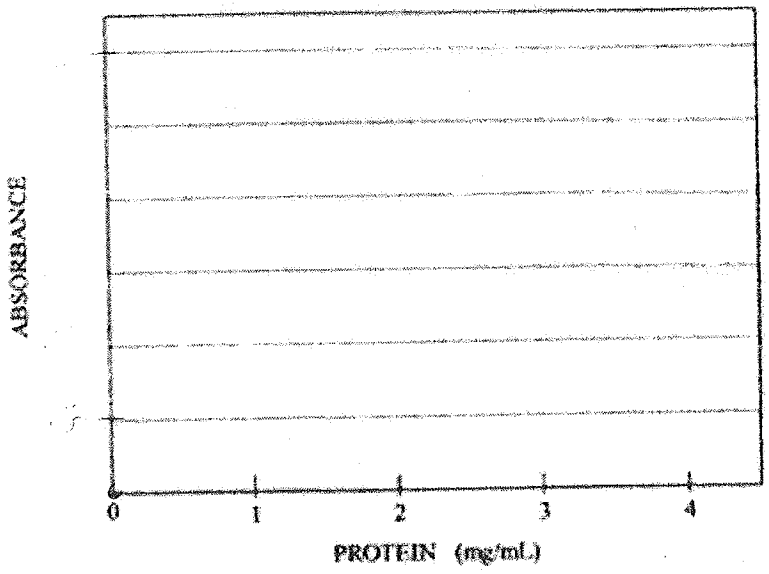
$.1)\ \frac{x}{.125} = \frac{2}{.125}$ $\quad 4)\ \frac{x}{.5} = \frac{2}{.125}$

Name _____; Section _____; Date _____

Table 3.3. Protein content for urine from different patients.

Patient:	1	2	3	4	5	6
Tube number:	P-1	P-2	P-3	P-4	P-5	P-6
Absorbance:						
Protein (mg/mL):						
from graph:						
calculated: *						

Standards:	B	S-1	S-2	S-3	S-4
Protein (mg/mL):	0	1	2	3	4
Absorbance:					



*Show sample calculation: