GENERAL BOTANY SPECIAL LECTURE - SPECTROPHOTOMETRY

I. Spectrophotometry as a tool for better understanding of biological molecules
- **A. Qualitative - different molecules have unique absorption spectra - i.e., chlorophyll, anthocyanin, lignin, protein, RNA, and DNA, demonstrate unique absorbance peaks**
- **B. Quantitative - different quantities of the same compound(s) exhibit different optical densities - i.e., increasing chlorophyll concentration increases absorbance values**
- **C. Kinetics - rate of reaction can be followed by monitoring specific absorbance of a product in a chemical reaction over time - i.e., enzyme kinetics (MDH, á-KG, PAL, HMG-CoA)**

II. General types of spectroscopic methods
- **A. Emission - no external radiation source is required; the sample itself emits a characteristic radiation, often after being excited by a flame**
- **B. Absorption - radiation beam (light) passes through and is altered by the sample**
- **C. Fluorescence - radiation induces the sample to emit a characteristic radiation which is measured with respect to the original radiation**

III. Main components of spectroscopic instruments
- **A. Stable source of radiation (usually some type of light)**
- **B. Wavelength selector that permits isolation of a restricted wavelength region**
- **C. Transparent container for holding the sample**
- **D. Radiation detector or transducer that converts radiant energy to a usable signal**
- **E. Signal processor and readout**

IV. Example of a specific instrument: HP8452A UV-VIS Diode-Array Spectrophotometer
- **A. Components include: a deuterium/tungsten lamp, source lens, shutter, sample compartment, spectrograph lens, slit, holograph grating, diode array, processor, and readout (see figure)**
- **B. How the spectrophotometer works**
  1. **Deuterium lamp emits UV radiation and tungsten lamp emits visible radiation.**
  2. **Radiation is received by the source lens which collimates it so that the beam passes directly though the sample.**
  3. **Shutter opens during measurement and closes after measurement to determine the relative difference between measuring and dark current.**
  4. **During measurement, beam passes through sample in the cuvette which may be regulated many different ways (temperature control, stirring, etc.).**
  5. **After passing through the sample, the beam passes through the spectrograph lens which directs the beam into the slit.**
  6. **The slit receives the beam and ensures that it is exactly the size of one of the photodiodes in the photodiode array. The slit immediately precedes the grating.**
  7. **The grating (which is a plate of glass with narrowly-spaced grooves) acts like a prism to separate the beam into all its component wavelengths and reflect it onto the diode array.**
  8. **Photodiodes, each with an assigned wavelength, make up the diode array. These diodes quantify specific radiation of beam components and report to signal processors.**
  9. **Processors interpret signals from photodiodes and report findings for eventual readout.**

Figure taken from:

HP 8452A Diode-Array
***Spectrophotometer Handbook*.**
1990. HP Part No. 08452-90002.

SUPPLEMENT TO EXERCISE 16: PHOTOSYNTHESIS

To be listed under "D. Spectrophotometry and Photosynthesis"

Operation of Spectrophotometer

Draw a rough sketch and identify the following components of the HP8452A UV-VIS Diode Array Spectrophotometer on display: 1) deuterium/tungsten lamp which emits UV/VIS radiation as the "source," 2) source lens and shutter which operate to collimate radiation and release it to the sample, respectively, 3) sample cuvette though which the beam passes, 4) spectrograph lens which refocuses the beam and directs it into the slit, 5) area in which you would find the slit, grating, and diode array, which focus, scatter, and quantify radiation levels, and 6) computer which, in part operates spectrophotometer and processes the data for visual analyses.

Simple Wavelength Scan

Obtain a two vials, one containing pure acetone and the other containing a crude chlorophyll extract in acetone. Using the menu-driven options on the HP 8452A Spectrophotometer Computer System, scan a blank sample of acetone and then scan the crude chlorophyll extract. Look closely as the scan report and compare to the absorption spectrum of chlorophyll in your book. Discuss the differences in what you obtained from the crude chlorophyll extract and what you see in the textbook. Be sure to be able to explain why the absorption peaks appear at about 450 and 675 nanometers and relatively few peaks occur between 500 and 600 nanometers on your spectrograph.

SUPPLEMENT TO EXERCISE 18: AEROBIC RESPIRATION

To be listed under "B. Respiratory Enzyme - Malate Dehydrogenase"

Specific components of respiration can be studied spectrophotometrically by detecting activities of enzymes involved. In this experiment, one of the enzymes of the Krebs Cycle, malate dehydrogenase (MDH), is studied by detection of NADH, which is produced during oxidation of malate to form oxaloacetate (OAA).

1. **Combine, in one container, the substrate solution: [1 mM NAD (cofactor), 5 F M antimycin A (special inhibitor), 1 mM deoxycholic acid (detergent), and 20 mM malate (substrate)].**

2. **Extract some MDH from root tissue by grinding the plant roots in a buffered solution (pH = 7.0) and filtering to reveal a clear, crude extract. Store on ice.**

3. **Combine, in a spectrophotometer cuvette, one part substrate with one part extract, and place cuvette in the spectrophotometer (which should already be standardized and scaled).**

4. **Observe an increase in absorbance at 340 nm. which is indicative of NADH production.**

Explain these results:

OPERATION OF SPECTROPHOTOMETER

Example: Determination of Chlorophyll Spectrum

1. **Turn on the system by pressing the master switch to the "on" position. The master switch is located on the outlet power strip.**

2. **Wait for the computer screen to display the password menu and listen for the spectrophotometer to perform several audible "clicks."**

3. **Type the password on the computer. The password is BIDLACK.**

4. **Once you get the General Menu, choose option #7 for the HP Spectrophotometer System.**

5. **Once you get the HP Spectrophotometer System Menu, choose the General Scanning option.**

6. **In the General Scanning mode, select "Files" (F6).**

7. **In the Files option, select "Recall Parameter File."**

8. **In the Parameter Files, select the file, "CHLORO.GSP."**

9. **Press the escape button until you return to the General Scanning mode.**

10. **Put a blank sample (acetone) into the cuvette, place in the spectrophotometer, and select "Measure Blank (F2) in the General Scanning mode. This is the "blank" absorption spectrum.**

11. **Be amazed with the absorption spectrum that is displayed. Note that there is lots of noise in the spectrum, but the scale indicates that this noise is insignificant.**

12. **Remove the blank sample, replace it with a sample containing chlorophyll (in acetone), and select the "Measure Sample" option (F1) at the bottom of the screen.**

13. **Be amazed with the absorption spectrum that is displayed. This is the absorption spectrum of chlorophyll.**

14. **Choose the "Hard Copy" option (F9) at the bottom of the screen and then choose the "Print Spectra" option that appears. The printer will begin printing what you've got on the screen.**

15. **Press the escape or exit (F10) button until you are back to the original menu.**

16. **Turn off the machine to conserve deuterium emission in the lamp.**