

Cryphonectria parasitica tendrils on chestnut tree bark (Photo: Ministry of Agriculture and Regional Developn Archive, Ministry of Agriculture and Regional Development, Bugwood.org)





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# Spectrophotometry and Beer's Law

B3 Summer Science Camp at Olympic High School

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Spectroscopy measures how matter interacts with radiation.

$$E = hv \qquad \lambda = \frac{c}{v}$$

- Radiation is characterized by frequency or wavelength.
  - Radiation passes through or bounces off matter resulting in a change in the number of photons (intensity), a frequency shift or a scatter pattern
- Absorbance
  - Interactions of photons with matter are frequency/wavelength dependent so monochromatic light (single-wavelength) is used.
  - The amount of light before it gets to your material (incident intensity) is decreased by the amount the material absorbs, so exiting light intensity is less than *incident* light intensity.
- Scattering
  - Photons *reflect* from the material coherent light will change direction
- Fluorescence
  - Interactions with molecules causes photons to shift to higher electron orbitals, energy is lost by photons as they decay back down

Absorbance: when light is *absorbed* by molecules the energy of the photon may promote an electron from a ground state to a new <u>orbital</u>.

- The energy provided has to match the energy difference in the orbitals, changing the state of the molecule.
  - This energy can be released by electron decay to the original state or a new state, with photon release (light is emitted at a new wavelength), or by transfer to another molecule or dissipation to the environment.
  - For proteins and nucleic acids, photons in the UV and near-UV/visible range have the right energy to be absorbed.

• Organic molecules subjected to UV-visible light have transitions from

- pi bonding to pi antibonding orbitals
- non-bonding to pi antibonding orbitals
- non-bonding to sigma anti-bonding orbitals.





## Beer-Lambert Law

• The law is an expression of a relationship:

 $A = \varepsilon C \ell$ 

- A is the absorbance
- $\epsilon$  is a constant for a given substance, if it is molar units them it is {Liters per mole per centimeter, or L- mol <sup>-1</sup> cm<sup>-1</sup>}
  - For nucleic acids  $\varepsilon_{260} = 50$  ml/ng-cm for dsDNA, 40 ml/ng-cm for RNA and 33 ml/ng-cm for ss DNA.
- C is the concentration (units are in moles/Liter)
- *l* is the path *l*ength that the light passes through the sample (converted to centimeters for whatever spectrophotometer you used, so units of the constant cancel out properly)

# Absorbance spectrum

• Biopolymers are complicated,





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# Absorbance spectrum

• Biopolymers have many vibrational energy levels so there are many closely-spaced individual absorption peaks that sum to a broad peak.



Measuring the changes in light as it interacts with matter is called spectrophotometry

• A spectrophotometer selects light of a determined wavelength from a source, passes it through a sample, and detects the number of photons (intensity) and/or frequency of the wavelength that reaches the detector.



### Absorption of light by DNA and Proteins

- DNA and proteins absorb light (photons) whose frequency is in the ultraviolet range (240-300nm).
- If some photons are absorbed going through a sample, fewer will emerge from the far side of a sample
- The intensity of the light (number of photons) will decrease on the far side.

**Transmittance**,  $T = P / P_0$ % **Transmittance**, %T = 100 T

Absorbance,

 $A = log_{10} P_0 / P$   $A = log_{10} l / T$   $A = log_{10} 100 / \% T$  $A = 2 - log_{10} \% T$ 



The law says that the fraction of light absorbed by each layer of solution is the same.

- Say that the fraction is 0.5 for each 0.2cm layer.
- Here is the data:

Path length / cm	0	0.2	0.4	0.6	0.8	1.0
%T	100	50	25	12.5	6.25	3.125
Absorbance	0	0.3	0.6	0.9	1.2	1.5



#### Is the law true everywhere?

• When the solution is too concentrated no light at all passes through.



# Averages of Measurements

- On the spectrophotometer you took multiple readings.
- Usually you take the mean or median in order to get the best *estimate* of accuracy.
  - The mean: add the replicate measurements together and divide by the number of measurements you took of that sample.
  - The median: take the middle value of the measurements (separating the top half and the bottom half this does not work if you only took 2 replicates)

### Finding the *range* of your measurements

- The smallest interval that contains all your data is the range (the highest to lowest value)
  - The variance shows how far the numbers are from the average (mean).
  - You calculate variance by subtracting the mean from each measurement and taking the square , summing these values and dividing by the number of values.
- The standard deviation is often used it is the *square root* of the variance
  - That way it has the same units as the measurements.

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