LICENCIATURA EM BIOLOGIA

DISCIPLINA

BIOQUÍMICA

Ano Lectivo de 2013/2014

8ª aula

Espectrofotometria

Introdução à espectrofotometria. A lei de Lambert-Beer.

Exemplo de aplicação ao caso das desidrogenases. Problemas.

Trabalho prático 1: quantificação de hidratos de carbono por métodos espectrofotométricos.

Trabalho prático 2: métodos espectrofotométricos quantitativos de determinação de proteína:

Métodos espectrofotométricos directos: Leitura das absorvâncias a 205 nm e a 280 nm.

Método de Lowry modificado: I-Preparação de curvas de calibração. II-Doseamento da proteína.

Material de apoio: protocolo do trabalho experimental.

INTRODUCTION

Spectroscopy
Science that addresses the interaction of matter with various types of radiation

Spectrometry / spectrometric methods
Group of analytical methods that are based on atomic and molecular spectroscopy
They deal with the measurement of the intensity of radiation with a photoelectric transducer

Spectrophotometry
Measurement of chemicals based on spectral information from a number of photons in a spectrophotometer

Photometry
Quantification by light intensity, not necessarily keeping the spectral information

Colorimetry
Quantification in a colorimeter

SPECTROSCOPY *VS.* SPECTROPHOTOMETRY

- Light can either be *transmitted* or *absorbed* by dissolved substances
- Presence & concentration of dissolved substances is analyzed by passing light through the sample
- Spectroscopes measure electromagnetic ***emission***
- Spectrophotometers measure electromagnetic ***absorption***

SPECTROPHOTOMETRY

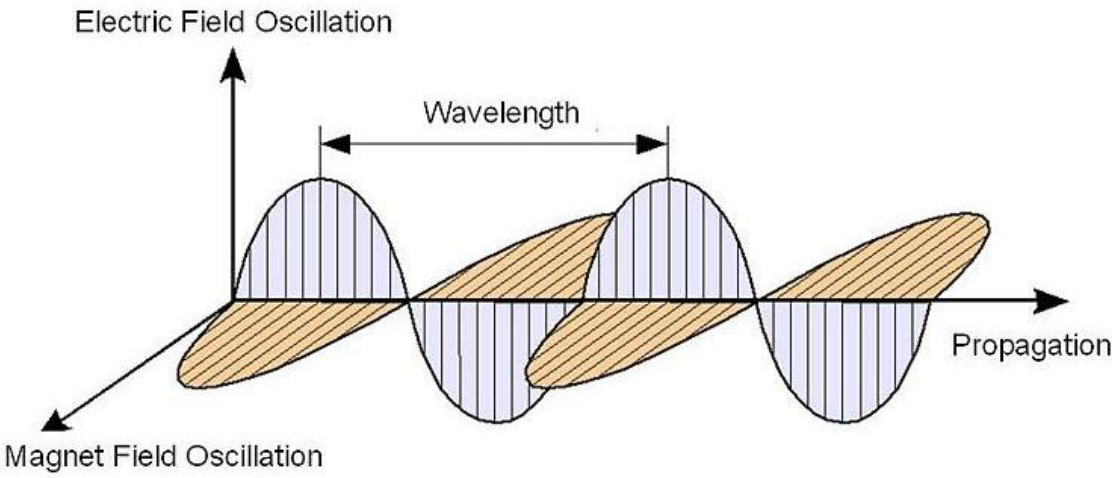
Electromagnetic Radiation = Light

What is Light?

1. Visible light is a particular kind of electromagnetic radiation
2. X-rays, UV, Infrared, Microwaves, and Radio waves are all light forms
3. Light is a form of Energy
4. All light travels in waves at “the speed of light” = c = 3 x 10^8 m/s

ELECTROMAGNETIC RADIATION

Two independent components: a the electric field and magnetic field oscillating in perpendicular phases

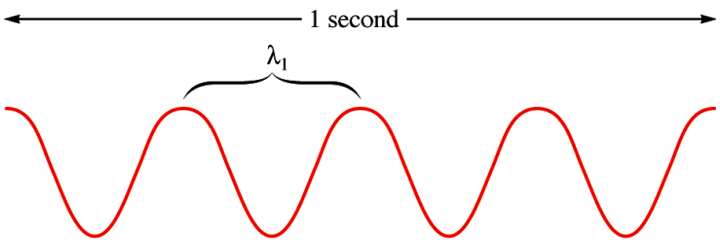


- The electric component is responsible for most of the phenomena: transmission / reflection / refraction and adsorption

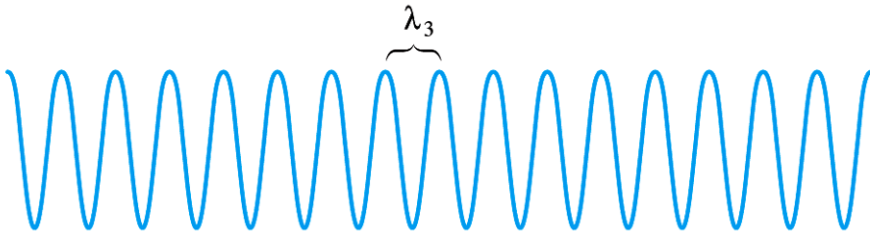
- The magnetic component is responsible for the absorption of radio waves

Characteristics of Light

1. **Wavelength** = distance between two peaks in a wave
   - *a.* λ (lambda) is the symbol
   - b. Meters = m is the unit
2. **Frequency** = number of complete waves passing a given point per second
   - *a.* ν (nu) is the symbol
   - b. Hertz = Hz = s^{-1} is the unit
3. **Amplitude** = *A* = measure of the intensity of the wave, "brightness"
4. The speed of light is constant: $c = \lambda \times \nu$
   - a. λ and ν are inversely proportional
   - b. If one increases, the other decreases
   - c. Their product is always the speed of light = $c = \lambda \times \nu = 3 \times 10^8$ m/s



ν_1 = 4 cycles/second = 4 hertz



ν_3 = 16 cycles/second = 16 hertz

PROPERTIES OF ELECTROMAGNETIC RADIATION

Electromagnetic radiation can be described by the classical model of a sinusoidal wave
-characteristic wavelength, speed, frequency and amplitude

The classical model explains the phenomena of emission and absorption of radiant energy

The application of a quantum model where the electromagnetic radiation beam is seen as a discrete particle layers photons, with energy proportional to the wavelength

The wave-particle duality is not exclusionary but rather complementary, helping to explain the behavior of cool electrons, protons and other elementary particles, which follow the principles of wave

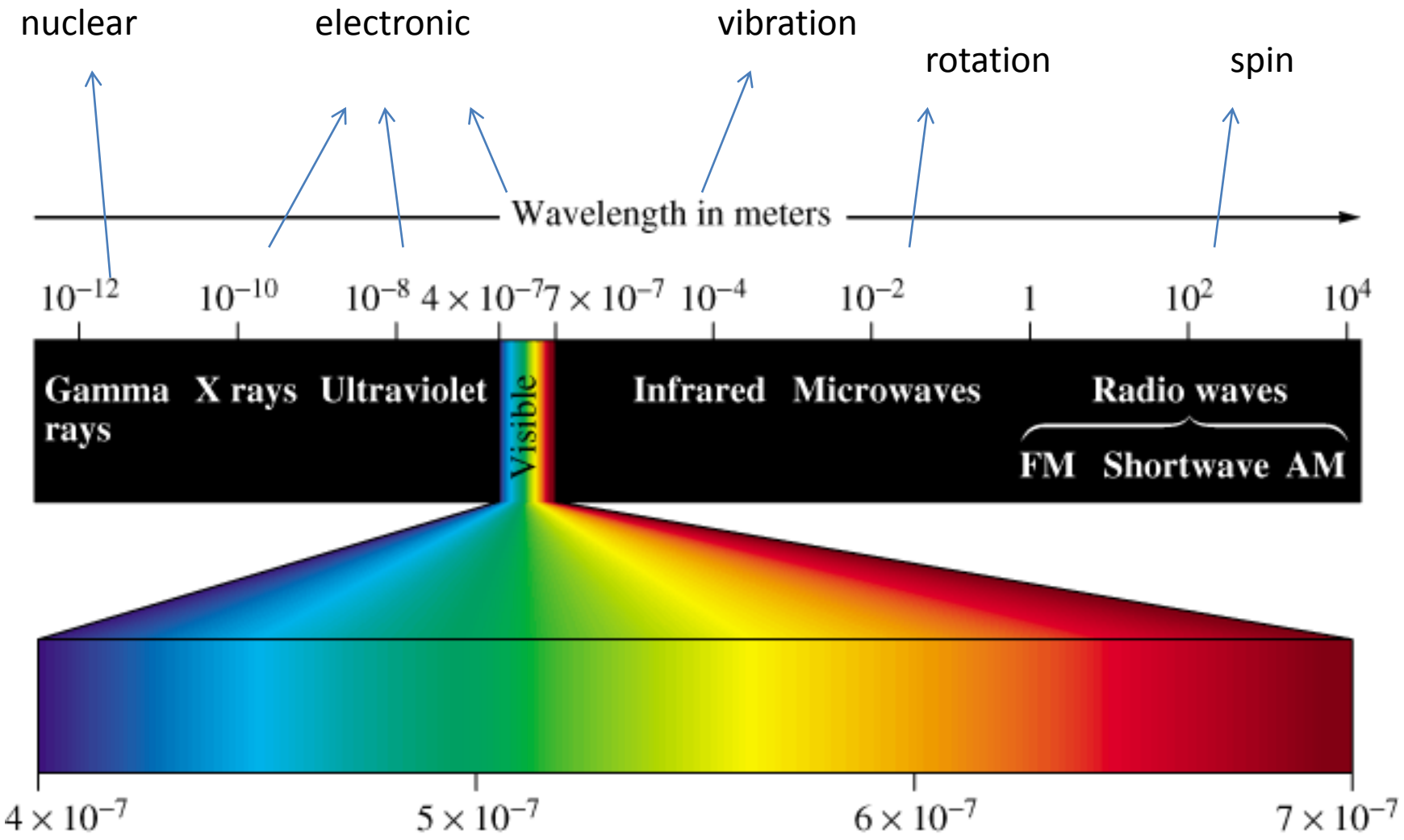
STATE ENERGY OF CHEMICAL SPECIES

Postulates of Quantum Theory:

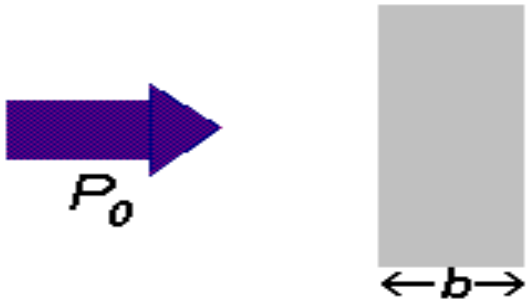
1. Atoms, ions and molecules can only exist in discrete energy states. Any change in this state, absorption or emission of energy, will be the amount of energy exactly equal to the difference between the two states

2. When atoms, ions or molecules absorb or emit radiation in the transition from one energy state to another, E0 and E1, the energy difference depends on the wavelength, λ, ν and the frequency of the radiation involved

ENERGETIC TRANSITIONS



Many compounds absorb ultraviolet (UV) or visible (Vis.) light.
The diagram below shows a beam of monochromatic radiation of radiant power P_0, directed at a sample solution. Absorption takes place and the beam of radiation leaving the sample has radiant power P.



The amount of radiation absorbed may be measured in a number of ways:

Transmittance, $T = P / P_0$

% Transmittance, $\%T = 100\ T$

Absorbance,

$$A = \log_{10} P_0 / P$$

$$A = \log_{10} 1 / T$$

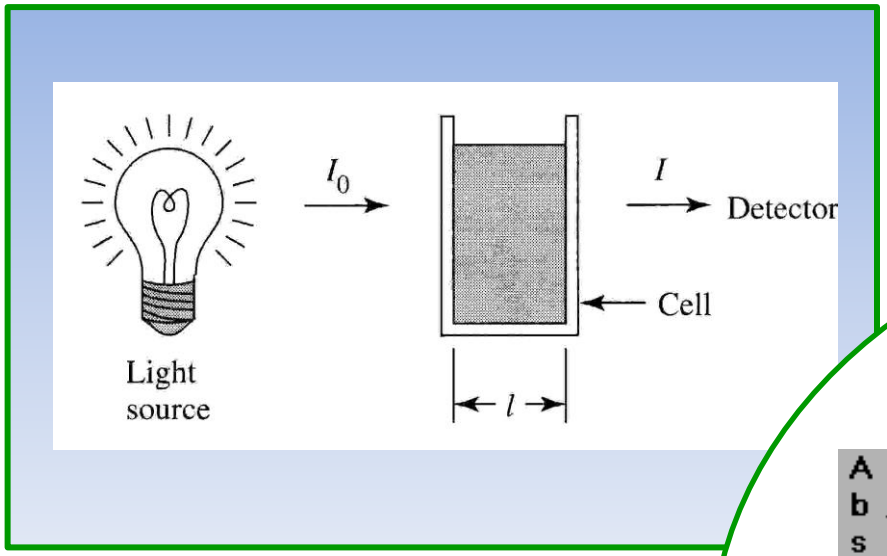
$$A = \log_{10} 100 / \%T$$

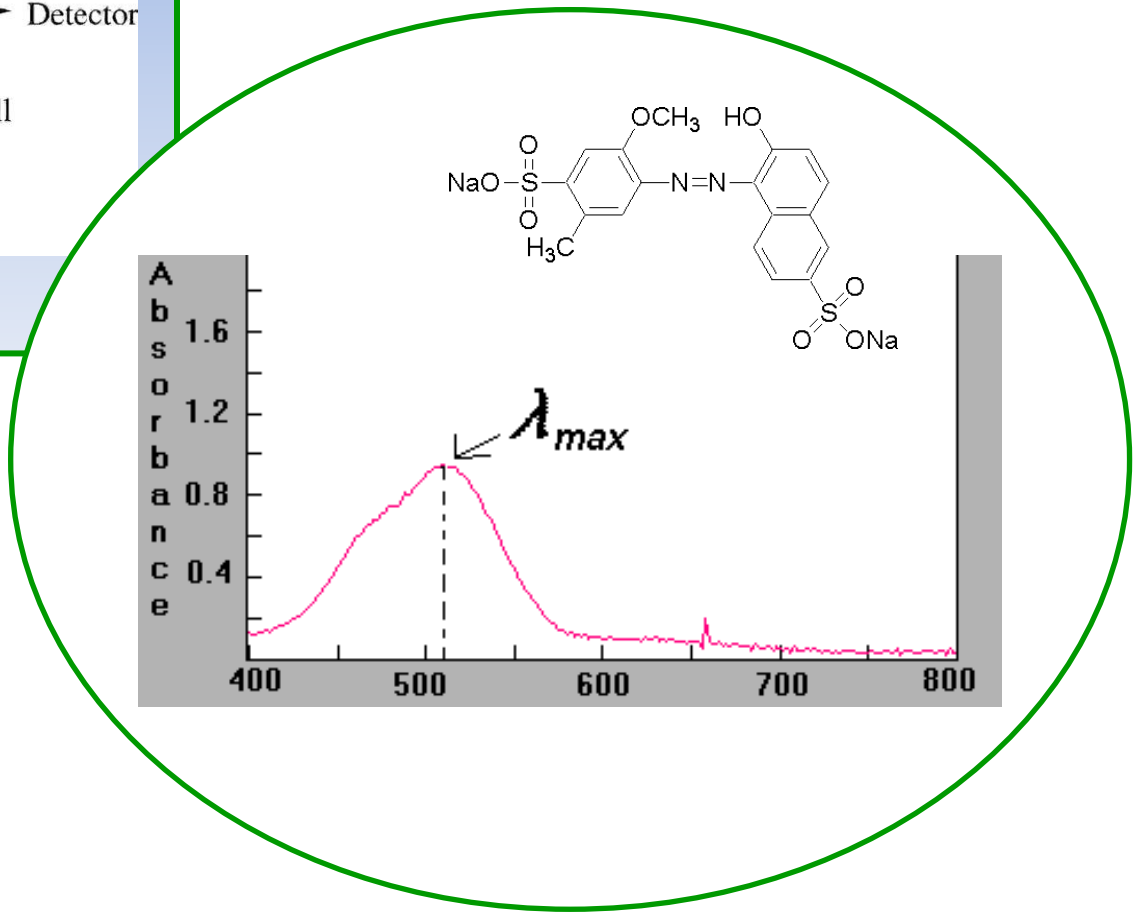
$$A = 2 - \log_{10} \%T$$

(easily calculate absorbance from percentage transmittance data.)

Spectroscopy = using light to investigate a compound

A. How the experiment works

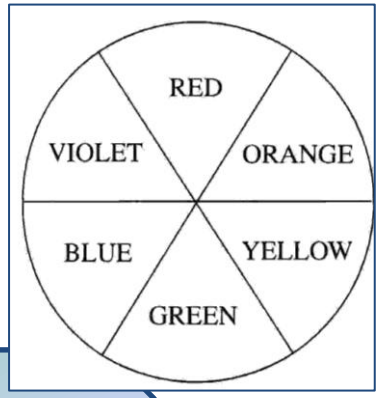




B. What color do we see?

a) What we see as the color of a compound is the complementary color to what the compound absorbs

b) Example: Absorbs red, we see green



Wavelength range(nm)	*Wave numbers (cm^{-1})*	*Color*	*Complementary color*
< 400	> 25,000	ultraviolet	
400-450	22,000-25,000	violet	yellow
450-490	20,000-22,000	blue	orange
490-550	18,000-20,000	green	red
550-580	17,000-18,000	yellow	violet
580-650	15,000-17,000	orange	blue
650-700	14,000-15,000	red	green
> 700	< 14,000	infrared	

The Beer-Lambert Law

1. Where does this law comes from:

   The law comes from a mathematician, Johan Heinrich Lambert, and a physicist and mathematician, August Beer. Lambert discovered the relationship between absorbance and path length, and Beer discovered the relationship between absorbance and concentration.

2. Why this law is useful:

   Given a path length, a plot relating absorbance and concentration will be a straight line. This makes analysis of solution easy.

Lambert's Law of Absorption

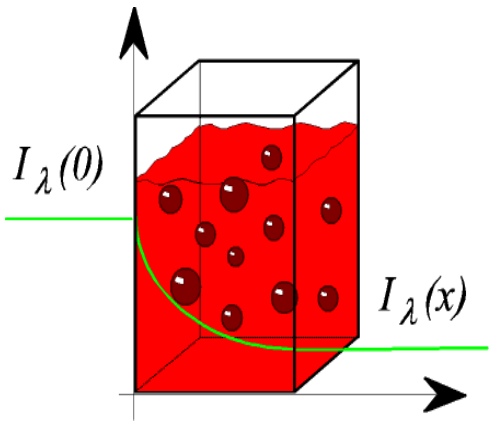
Lambert described how intensity changes with distance in an absorbing medium.

- The intensity I_0 if a beam of light decreases exponentially as it passes though a uniform absorbing medium with the linear decay constant α.

  Restatement: In a uniform absorbing medium, the intensity of a beam of light decreases by the same proportion for equal path lengths traveled.

- The linear decay constant α is a characteristic of the medium. It has units of reciprocal length. α is the path length over which the intensity is attenuated to $1/e$.

Johann Heinrich Lambert
1728-1777



$$I = I_0 e^{-\alpha x}$$

The distance traveled through the medium is called the *path length*.

$$\mathrm{d}I = -\alpha I \,\mathrm{d}x \qquad \frac{\mathrm{d}I}{\mathrm{d}x} = -\alpha I$$

$$I = I_0 e^{-\alpha x} \qquad \frac{I}{I_0} = e^{-\alpha x}$$

Lambert's Law of Absorption (base 10)

Typically base 10 is used in photometry.

$$I = I_0 e^{-\alpha x} = I_0 10^{-kx} \qquad k = \alpha \ln 10$$

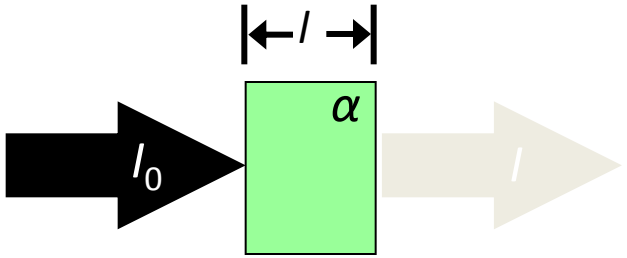
$$\frac{I}{I_0} = e^{-\alpha x} = 10^{-kx}$$

k is the path length over which the intensity is attenuated to 1/10.

$$\boxed{\frac{I}{I_0} = 10^{-kx}}$$

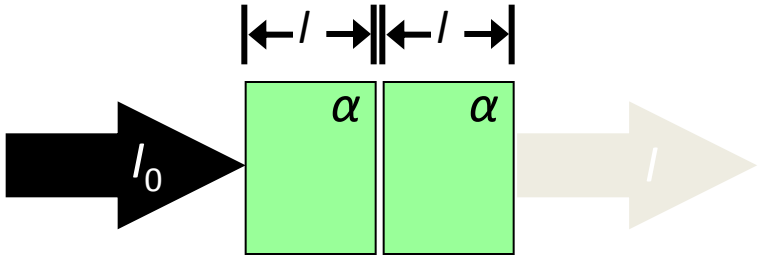
Lambert's Law Example

If *one slab* of absorbing material of thickness *l* reduces the intensity of a beam of light to *half.*



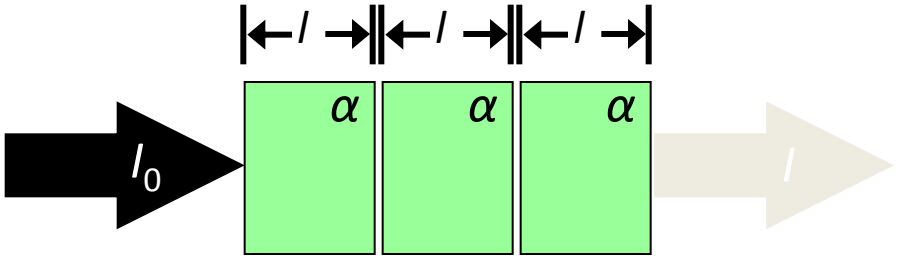
$$\frac{I}{I_0} = 10^{-kl} = \frac{1}{2}$$

Then *two slabs* of the same absorbing material will then reduce the intensity of a beam of light to *one quarter*.



$$\frac{I}{I_0} = 10^{-k2l} = \left(\frac{1}{2}\right)^2 = \frac{1}{4}$$

And *three slabs* will reduce the intensity of a beam of light to *one eight*.



$$\frac{I}{I_0} = 10^{-k3l} = \left(\frac{1}{2}\right)^3 = \frac{1}{8}$$

Beer's Law

Beer found that Lambert's linear decay constant *k* for a solution of an absorbing substance is linearly related to its concentration *c* by a constant, the absorptivity *ε*, a characteristic of the absorbing substance.

Restatement: The linear decay constant *k* is linear in concentration *c* with a constant of proportionality *ε*.

(August Beer, 1825-1863)

$$\boxed{k = \varepsilon c}$$

Typical units are: *k* cm^{-1}; *c* M (moles/liter); *ε* $M^{-1}cm^{-1}$

ABSORPTIVITY

A colored absorber has an absorptivity that is dependent on wavelength of the light $\varepsilon(\lambda)$.
The absorptivity is the fundamental property of a substance. This is the property that contains the observable spectroscopic information that can be linked to quantum mechanics (also see absorption cross section.)

DEFINITION

E = Molar Extinction Coefficient ---- Extinction Coefficient of a solution containing 1g molecule of solute per 1 liter of solution

Beer's Law

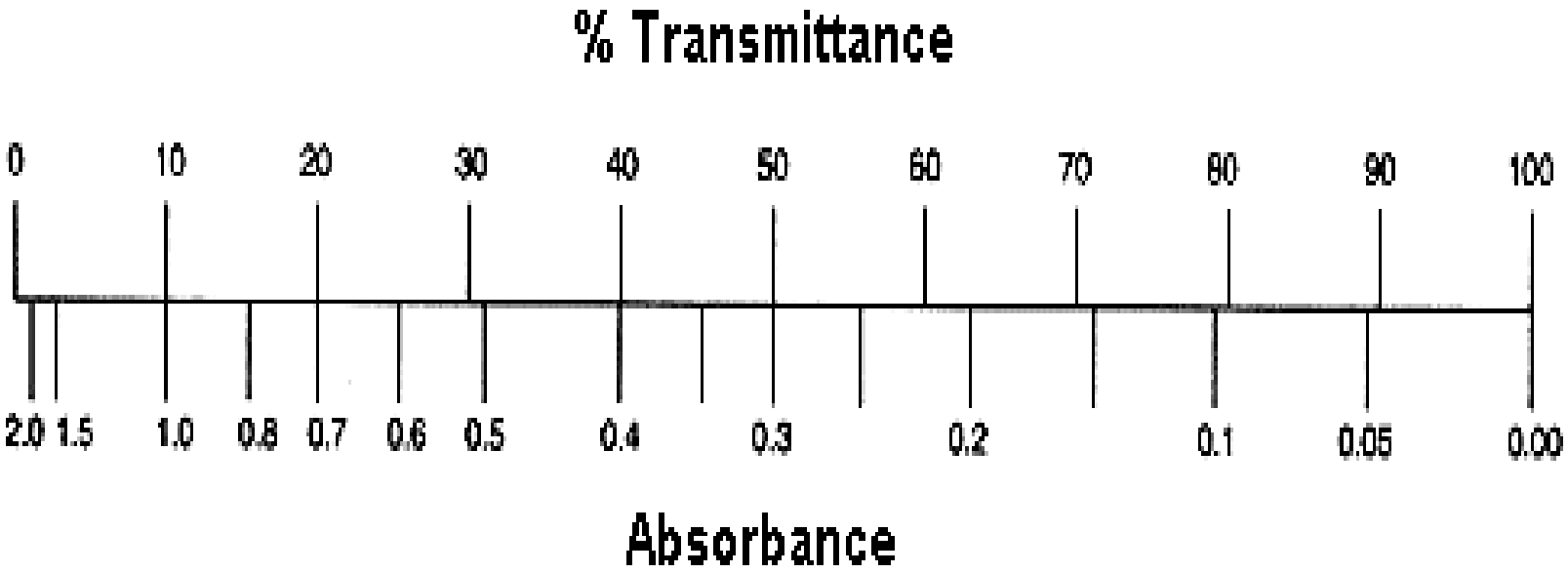
- The intensity of a ray of monochromatic light decreases exponentially as the concentration of the absorbing medium increases.

- More dissolved substance = more absorption and less transmittance

Graphical Relationship

- % transmission and % absorption are not linearly related to concentration
- For a graph to be useful, a straight line is needed

The relationship between absorbance and transmittance



On most of the diagrams you will come across, **the absorbance ranges from 0 to 1**, but it can go higher than that.

An absorbance of 0 at some wavelength means that no light of that particular wavelength has been absorbed. The intensities of the sample and reference beam are both the same, so the ratio P_o/P is 1. Log10 of 1 is zero.

An absorbance of 1 happens when 90% of the light at that wavelength has been absorbed - which means that the intensity is 10% of what it would otherwise be.

In that case, P_o/P is 100/I0 (=10) and log10 of 10 is 1.

Photometric Quantities

In photometry we **measure** the *intensity* of light and characterize its change by and object or substance. This change is typically expresses as *percent transmittance* or *absorbance*.

Transmittance (T)

Frequently when your primary interest is the light beam

$$T = \frac{I}{I_0}$$

usually given in percent

Absorbance (A) (AKA optical density, O.D.)

Used almost exclusively when your interest concerns the properties of the material

$$A = -\log\left(\frac{I}{I_0}\right) = -\log T$$

by convention, base 10 logs are used

Beer-Lambert Law

Lambert's and Beer's Laws are combined to describe the attenuation of light by a solution. It is easy to see how the two standard photometric quantities can be written in terms of this law.

$$I = I_0 10^{-\varepsilon c l}$$

Transmittance

$$T = \frac{I}{I_0}$$

$$T = 10^{-\varepsilon c l}$$

Absorbance

A=log 1/T = log I_0/I

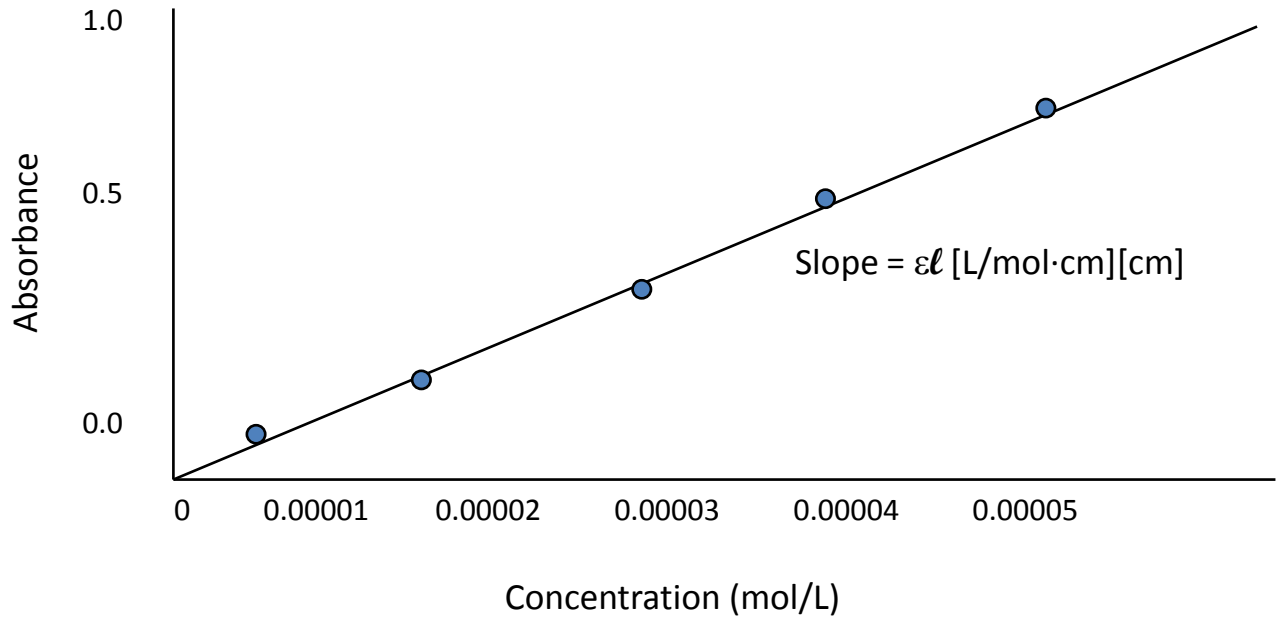
$$A = -\log\left(\frac{I}{I_0}\right) = -\log T$$

$$A = \varepsilon c l$$

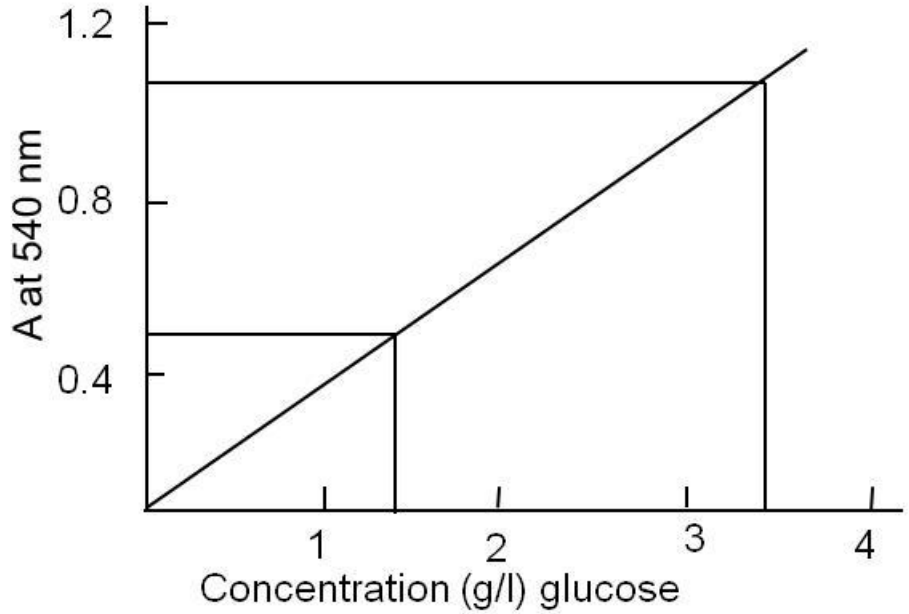
Beer-Lambert's Law

$A = \varepsilon lc$ $(y = mx + b)$ describes the absorption of light in a solution

- l = the length of the cell containing the solution, usual 1 cm
- c = concentration in mol/L = M
- ε = Molar extinction coefficient = constant for a given molecule at a given wavelength of light = how well the molecule absorbs light



SPECTROMETRIC ANALYSIS USING STANDARD CURVES



Avoid very high or low absorbencies when drawing a standard curve. The best results are obtained with $0.1 < A < 1$. Plot the Absorbance vs. Concentration to get a straight line

NEVER extrapolate beyond point known where becomes non-linear.

What is the significance of the molar absorbtivity, ε ?

$$A = \varepsilon lc :$$

$$\varepsilon = A / lc$$

"ε is a measure of the amount of light absorbed per unit concentration". Molar absorbtivity is a constant for a particular substance, so if the concentration of the solution is halved so is the absorbance

Chemical A: 100,000 L/mol/cm, which is in a solution in a 1 cm pathlength cuvette and gives an absorbance of 1 .

Chemical B: 20 L/ mol/ cm which is in solution in a 1 cm pathlength cuvette and gives an absorbance of 1.

$\varepsilon = 1 / lc$

Therefore, concentration of A is $1 / 100{,}000 = 1 \times 10^{-5}$ mol/L

concentration of B is 1 / 20 = 0.05 mol/ L

A compound with a high molar absorbtivity is very effective at absorbing light (of the appropriate wavelength), and hence low concentrations of a compound with a high molar absorbtivity can be easily detected.

Lambert-Beer's law deviations

-Deviations in absorptivity coefficients at high concentrations (> 0.01M) due to electrostatic interactions

-Scattering of light due to particulates in the sample

-Fluorescence or phosphorescence of the sample

-Changes in refractive index at high analyte concentration

- Non-monochromatic radiation

INSTRUMENTATION

- **Spectrometer:** measures I vs λ.
  Simply measures the spectrum of the light (e.g. emission spectroscopy).
- **Spectrophotometer:** measures I/I_0 vs λ.
  Measures how the sample *changes* the spectrum of the light (e.g. transmission, reflection, scattering, fluorescence).

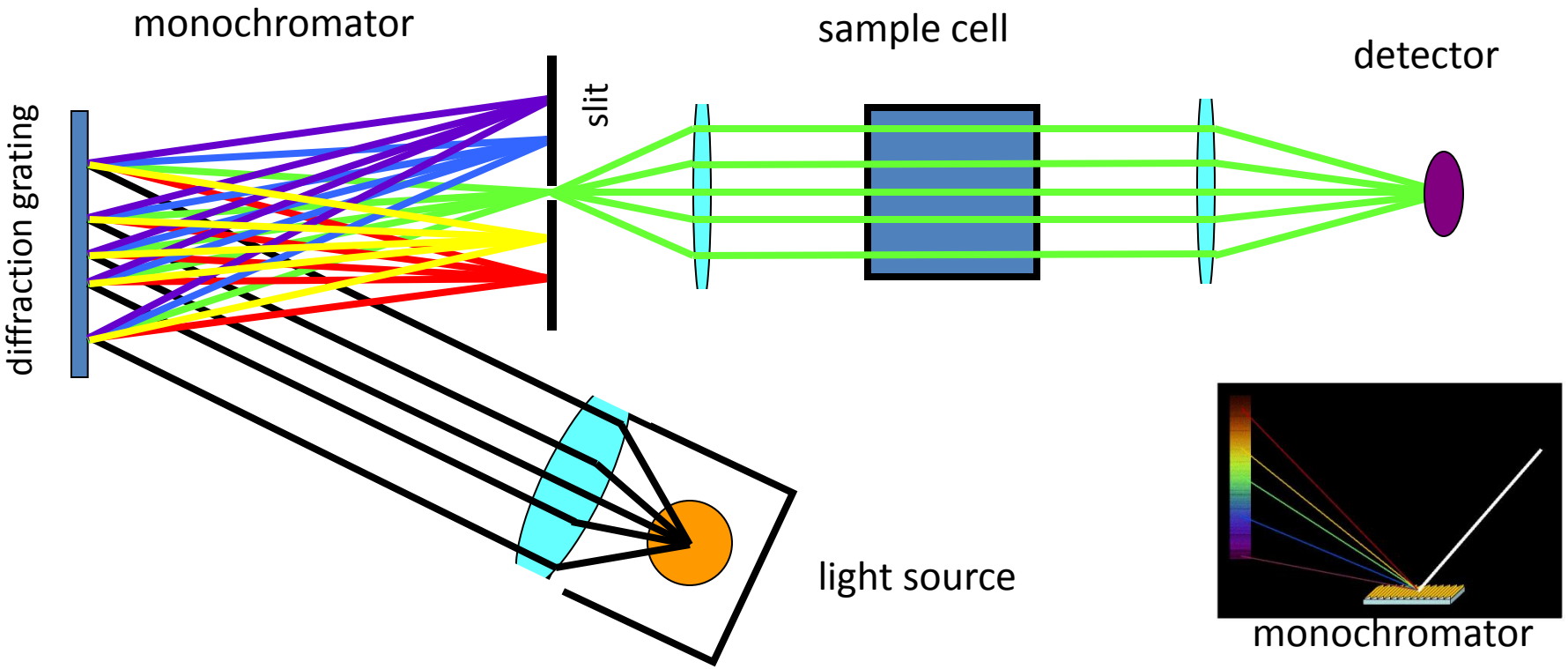
All spectrophotometers contain a spectrometer.

- **-meter:** the detector is electronic
- **-graph:** light intensity recorded on film
- **photometer:** measures I/I_0 without λ selection.

The Spectrophotometer

Measures absorbance as a function of wavelength

Components: light source, monochromator, sample cell, detector, optical system.



RADIATION SOURCE

Should be chosen from an energy source that does not vary over a wide range of wavelengths.

Thus we have:

Hydrogen or deuterium lamps

Used in the **ultraviolet region** of the spectrum (160nm to 350nm).

Bulbs with tungsten filaments

Used in the **visible region** (350nm to 800nm).

Argon or xenon lamps

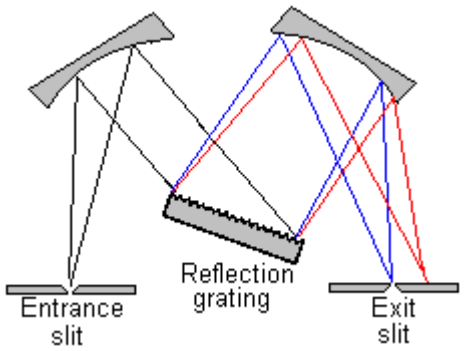
Used when you need a high intensity, emitting a continuous band of radiation between 200nm and 1000nm.

WAVELENGTH SELECTOR (MONOCHROMATOR)

All monochromators contain the following component parts;

- An entrance slit
- A collimating lens
- A dispersing device (usually a prism or a grating)
- A focusing lens
- An exit slit

Polychromatic radiation (radiation of more than one wavelength) enters the monochromator through the entrance slit. The beam is collimated, and then strikes the dispersing element at an angle. The beam is split into its component wavelengths by the grating or prism. By moving the dispersing element or the exit slit, radiation of only a particular wavelength leaves the monochromator through the exit slit.



Czerney-Turner grating monochromator

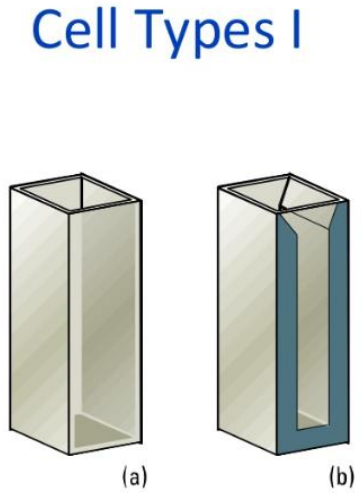
SAMPLE CELLS

UV Spectrophotometer

Quartz (crystalline silica)

Visible Spectrophotometer

Glass, plastic



DETECTORS

Any photosensitive device can be used as a detector of radiant energy. The photocell and phototube are the simplest photodetectors, producing current proportional to the intensity of the light striking them.

- A photoemissive cathode (emits electrons when struck by photons)
- Several dynodes (emit several electrons for each electron striking them)
- An anode
- Produces an electric signal proportional to the radiation intensity
- Signal is amplified and made available for direct display
- A sensitivity control amplifies the signal

PMT

CCD/CID

Photodiode

Thermocouple

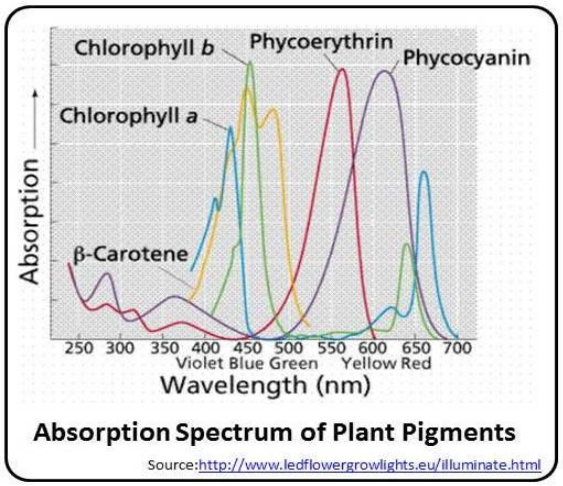
MCT

Pyroelectric detector

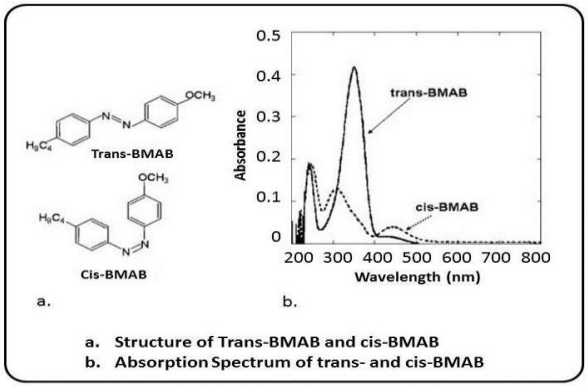
SPECTROPHOTOMETRY

is employed in a broad range of biology and biotechnology applications:

a. Quantitative analysis (Identification of the concentration of the unknown substance)
b. Qualitative analysis (Identification of an unknown substance)
c. Enzyme assays
d. Control of purification
e. Study of cis-trans isomerism
f. Quantification of organic or inorganic compounds in solutions
g. Turbidimetry
h. Test for rates of photosynthesis
i. Food safety analysis
j. Forensic analysis
k. Blood analysis
l. Disease diagnosis/ pathological states
m. Residue pesticide analysis
n. Measure the growth of bacterial culture
o. etc

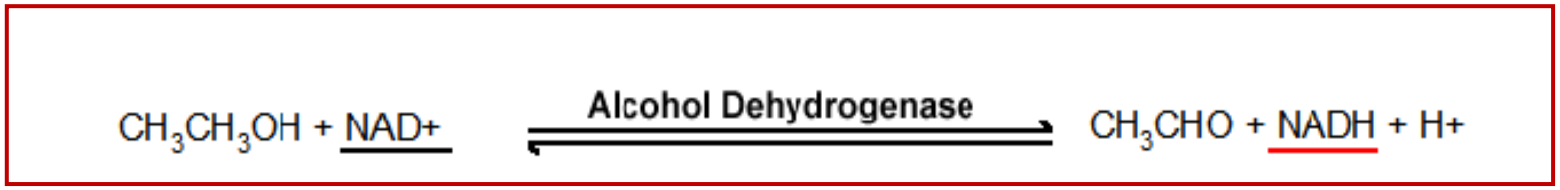


Absorption Spectrum of Plant Pigments
Source:http://www.ledflowergrowlights.eu/illuminate.html



a. Structure of Trans-BMAB and cis-BMAB
b. Absorption Spectrum of trans- and cis-BMAB

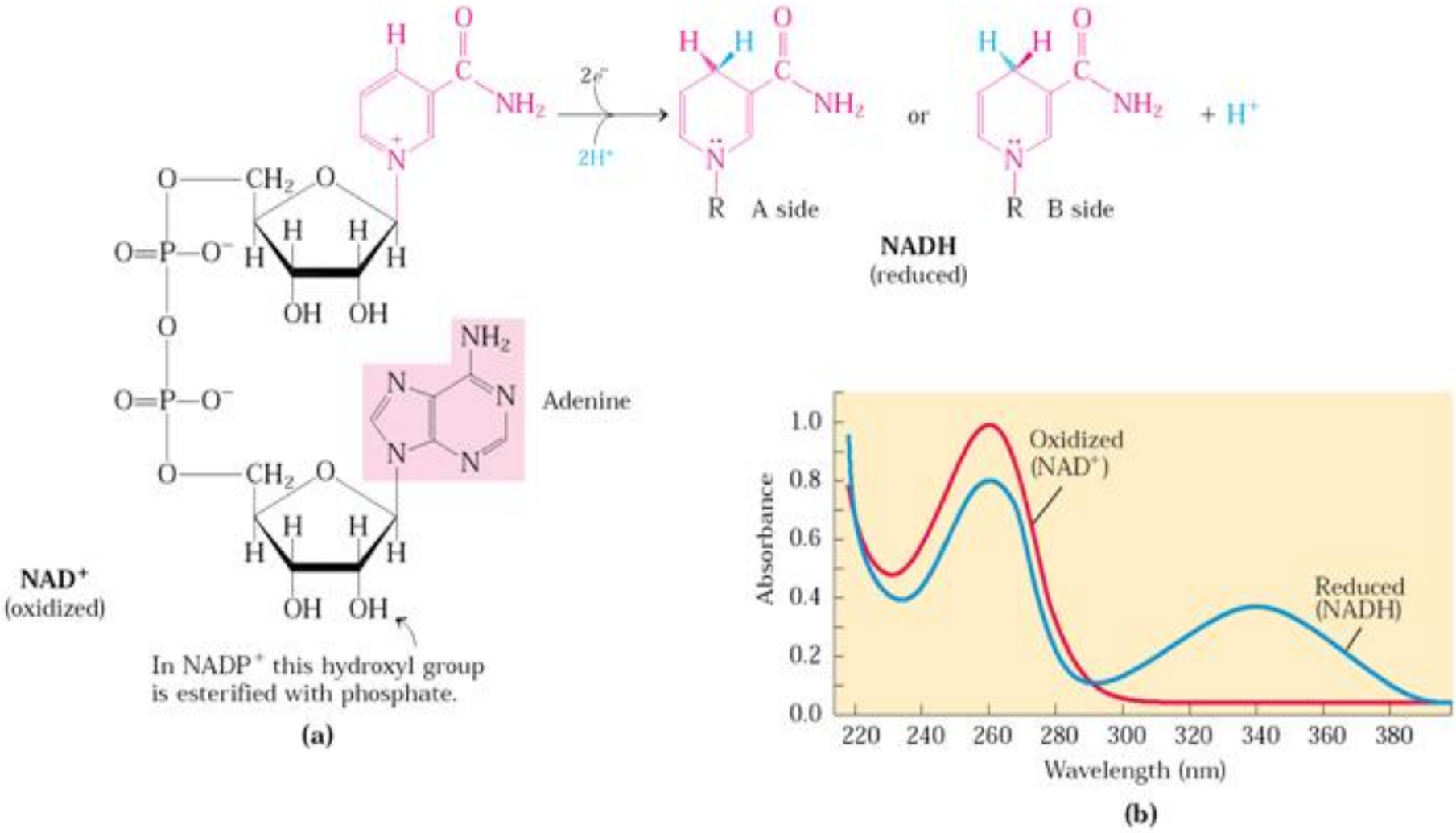
EXAMPLE



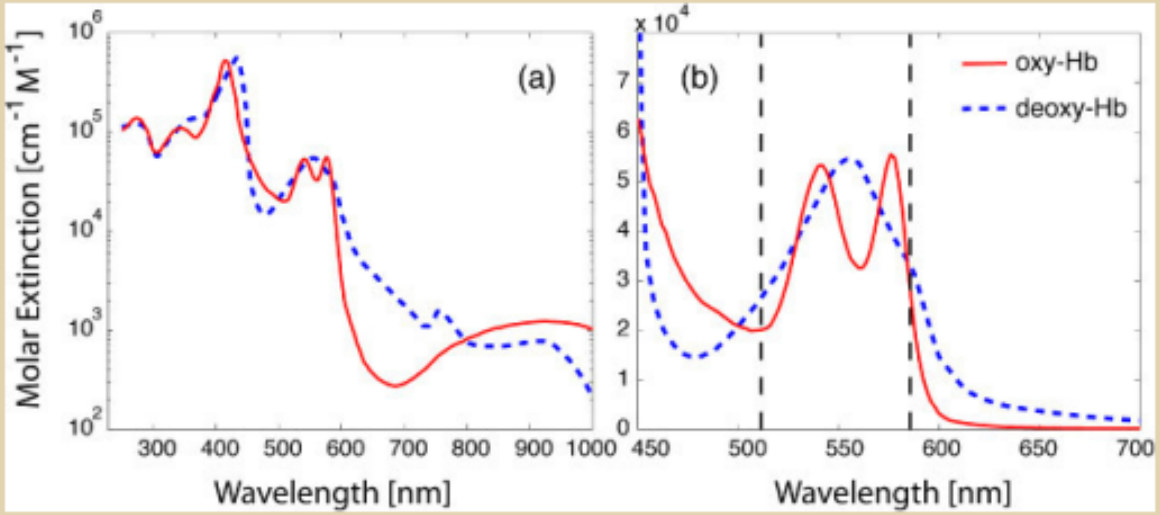
Both NAD^+ and NADH absorb strongly in the ultraviolet due to the adenine base.

Peak absorption of NAD^+ is at a wavelenght of 259 nanometers (nm), with an extinction coefficient of 16,900 $M^{-1}cm^{-1}$. NADH absorbs at higher wavelengths, with a second peak in UV absorption at 339 nm, with an extinction coefficient of 6,220 $M^{-1}cm^{-1}$.

This difference in the ultraviolet absorption spectra between the oxidized and reduced forms of the coenzymes at higher wavelengths makes it simple to measure the conversion of one to another in enzyme assays – by measuring the amount of UV absorption at 340 nm using a spectrophotometer.


2e⁻
2H⁺
A side
or
B side
+ H⁺
NADH
(reduced)
Adenine
NAD⁺
(oxidized)
In NADP⁺ this hydroxyl group
is esterified with phosphate.
(a)
Absorbance
1.0
0.8
0.6
0.4
0.2
0.0
Oxidized
(NAD⁺)
Reduced
(NADH)
220
240
260
280
300
320
340
360
380
Wavelength (nm)
(b)

EXAMPLE

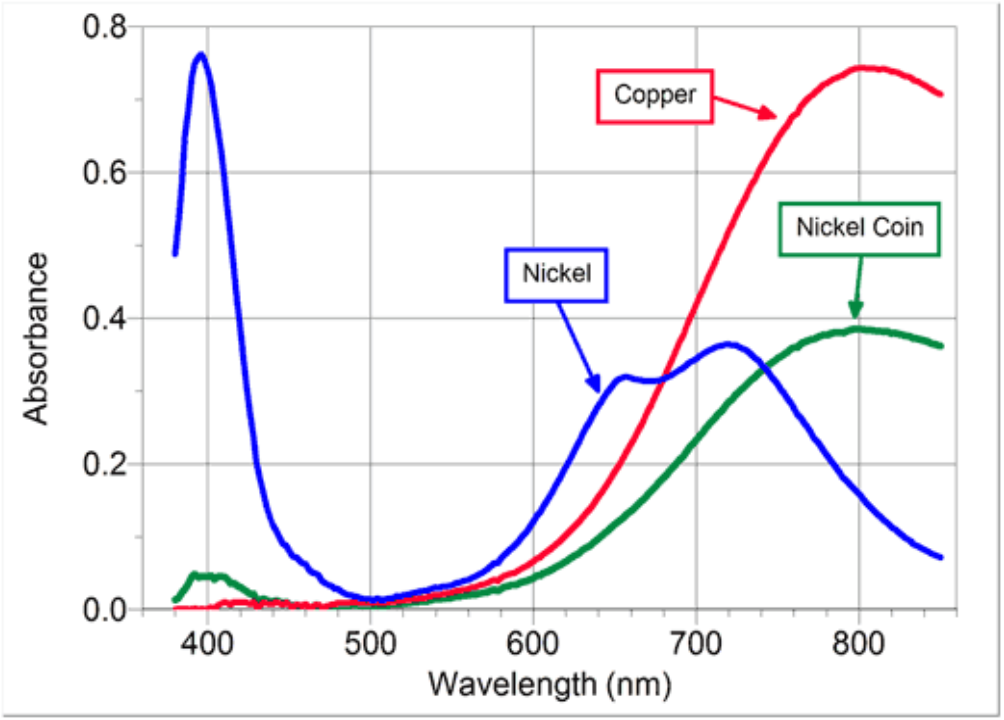


g002: Molar extinction coefficients of oxy-/deoxy- Hb over a large spectral range (a), and across the visible region of the spectrum (b). The dotted black lines in (b) delineate the region where the oxy- and deoxy- Hb coefficients exhibit the greatest dissimilarity (correlation R~0). Data compiled by Prahl [15].

Mentions: For detection, the grating in the system's imaging spectrograph is set to operate in the visible region of the spectrum, centered about 575 nm with a bandwidth of ~250 nm. As seen in Fig. 2(a)Fig. 2

Assessing hemoglobin concentration using spectroscopic optical coherence tomography for feasibility of tissue diagnostics

EXAMPLE



A spectrometer was used to analyse the metals in a United States five-cent coins, by capturing the absorption spectra of a nickel standard solution, a copper standard solution, and solution containing part of a U.S. five-cent coin.

EXAMPLE

With the naked eye, it is obvious that beer ranges in color from a translucent light yellow color and to a dark brown, almost black opaque color. The color of finished beer is determined by a multitude of different factors in the chemistry of its makeup and ingredients; which makes it difficult to predict in advance.

λ= 430 nm, scale ranging from 2 to 40, to grade its color intensity

PROBLEMAS

· **Problem** 1

A suspension of bacteria containing 400 mg dry weight per liter has an absorbance of 1.00 in a 1 cm cuvette at 450 nm. What is the cell density in a suspension that has a transmission of 30% in a 3 cm cuvette?

Solution

First calculate the absorbance of the suspension in a 1 cm cuvette.

$$A_{3\,\mathrm{cm}} = \log \frac{I_0}{I} = \log \frac{100}{30} = \log 3.333 = 0.523$$

$$A_{1\,\mathrm{cm}} = \frac{A_{3\,\mathrm{cm}}}{3} = \frac{0.523}{3} = 0.1743$$

Because we know that an A of 1.00 is equivalent to 400 mg/liter of bacterial cells, the density equivalent to an A of 0.174 can be determined by simple proportions.

$$\frac{1.00\ A\ \text{unit}}{400\ \text{mg/liter}} = \frac{0.1743\ A\ \text{unit}}{X\ \text{mg/liter}}$$

X = 69.7 mg/liter

An alternative way of solving the problem is to define a specific absorption coefficient for the bacteria.

If an A of $1.00 \triangleq 400$ mg/liter bacteria, calculate the A of 1 g/liter bacteria.

$$\frac{1.00\ A \text{ unit}}{0.40 \text{ g/liter bacteria}} = \frac{a_s}{1.0 \text{ g/liter bacteria}}$$

$$\boxed{a_s = 2.5}$$

Now use the usual formula.

$$A = (a_s)(c_{\text{g/liter}})(l_{\text{cm}}) \qquad 0.523 = (2.5)(c_{\text{g/liter}})(3)$$

$$c_{\text{g/liter}} = \frac{0.523}{7.5} = 0.0697 \text{ g/liter}$$

$$\boxed{c = 69.7 \text{ mg/liter}}$$

· **Problem** **2**

The specific absorption coefficient ($a_{1\%}^{1\,cm}$) of a glycogen-iodine complex at 450 nm is 0.20. Calculate the concentration of glycogen in a solution of the iodine complex, which has an absorbance of 0.38 in a 3 cm cuvette.

Solution

$$A = a_{1\%}^{1\,\mathrm{cm}}\, c_{\%} l_{\mathrm{cm}} \qquad 0.38 = (0.20)(c_{\%})(3)$$

$$c_{\%} = \frac{0.38}{(0.2)(3)} = \frac{0.38}{0.6}$$

$$\boxed{c = 0.633\%}$$

Problem 3 A solution of purified DNA isolated from *Escherichia coli* gives an absorbance of 0.793 at 260 nm in a 1-cm cell at pH 4.5. If $E_{1\text{ cm}}^{1\%}$ is 197, calculate the concentration of the solution in milligrams per milliliter.

Solution:

$$A = E^{1\%}_{1\,\mathrm{cm}} \cdot c$$

since b is understood to be 1 cm. Solving for c we obtain

$$c = \frac{A}{E^{1\%}_{1\,\mathrm{cm}}}$$

Substitution gives

$$c = \frac{0.793}{197} = 4.03 \times 10^{-3}\%$$

A $4.03 \times 10^{-3}\%$ solution contains 4.03×10^{-3} g per 100 ml. Thus we have 4.03×10^{-5} gm ml^{-1} or 4.03×10^{-2} mg ml^{-1}.

HOMEWORK

1- Uma amostra de uma substância colorida, que se sabe seguir a lei de Beer-Lambert, apresenta uma transmitância de 80%, quando medida numa cuvette de 1 cm. Calcule a % de transmitância para uma solução de concentração duas vezes superior, medida na mesma cuvette.
R= 64%

2- O coeficiente de extinção molar de uma certa substância é 14.000 $M^{-1}cm^{-1}$ no comprimento de onda do seu máximo de absorção. Calcular a molaridade dessa substância que pode ser medida no espectrofotómetro com uma cuvette de 1 cm, para uma absorvência de 0,850.
R= 6,07 $x10^{-5}$ M

3- Expresse as seguintes absorvências em termos de percentagens de transmitância:

a) 0,051 b) 0,918 c) 0,379 d) 0,261

4- Converta os seguintes dados de transmitância em absorvências:

a) 25,5% b) 0,567 c) 32,8% d) 3,58

5- Uma solução contendo 4,48 g/L de $KMnO_4$ tem uma transmitância de 0,309 numa cuvette de 1,00 cm a 520 nm. Calcule a absortividade molar do $KMnO_4$.
R= 0,114 $M^{-1}cm^{-1}$

6- Uma alíquota de 50,0 mL de água potável é tratada com um excesso de KSCN e diluída para 100,0 mL. Calcule a concentração de ferro se a solução diluída forneceu uma absorbância de 0,507 a 580 nm, quando medido numa cuvette de 1,50 cm (ε = 7000 $M^{-1}cm^{-1}$). A estequiometria do complexo é 1:1.
R= 9,66 x10^{-5} M

7- Calcule o coeficiente de extinção molar (E) a 351 nm para a cianocobalamina (vitamina B12) em 0,1 M de solução tampão fosfato, pH 7,0, a partir dos seguintes dados (cuvette de 1cm):

Solução	C x 10^5 M	I_0	I
A	2,23	100	27
B	1,90	100	32

R= E_A= 1,66 x10-5 $M^{-1}cm^{-1}$ / E_B= 1,63 x10-5 $M^{-1}cm^{-1}$

8- Uma solução padrão foi adequadamente diluída para fornecer as concentrações de ferro mostradas abaixo. O complexo ferro(II)-1,10-fenantrolina foi então preparado de alíquotas de 25,0 mL dessas soluções. As absorvências seguintes foram medidas a 510 nm:

Concentração de Fe(II) nas soluções padrão (mg/mL)	Absorvência, A (cuvettes de 1,00 cm)
2,00	0,164
5,00	0,425
8,00	0,628
12,00	0,951
16,00	1,260
20,00	1,582

a) Construa uma curva de calibração a partir desses dados;
b) Calcule uma equação da recta utilizando regressão linear;
c) Calcule a concentração de ferro em alíquotas de 25,0 mL de água subterrânea e cujas absorvências foram:

Amostra 1: A_1= 0,143 **R=** 1,64 mg/mL

Amostra 2: A_2= 0,068 **R=** 0,68 mg/mL

9- O coeficiente de extinção molar (E) da riboflavina (vitamina B2) é 3 $x10^{3}$ Lcm^{-1} mol^{-1}. Se a leitura da absorvência a 350 nm de uma solução contendo riboflavina, for 0,9 usando uma cuvette de 1 cm, qual é a concentração de riboflavina na amostra?

R= 0,3 $x10^{-3}$ M

10- Sabendo que a concentração do composto Y é 2 $x10^{4}$ $molL^{-1}$ e que a absorvência da sua solução a 300 nm, usando uma cuvette de quartzo de 1 cm, é de 0,4. Qual é o coeficiente de extinção molar do composto Y?

R= 0,2 $x10^{-4}$ $M^{-1}cm^{-1}$

FIM