

Spectrophotometer

A spectrophotometer is an instrument that measures the amount of light that can pass through a solution. It is apparent that less light is allowed to pass through a highly turbid or colored solution than through a clear solution. Spectrophotometer is the device that can quantify the amount of light transmitted through solutions.

Inside a spectrophotometer, light is focused through a lens system to an entrance slit. The light rays are refocused by a second lens onto an exit slit. Between the second lens and the exit slit is a monochromatic grating which separates the white light into its component wavelengths in much the same fashion as a prism. By proper rotation of the monochromatic grating, specific light wavelengths may be passed on through the exit slit to a photocell. This cell is connected directly to a galvanometer which translates the electrical output of the activated photocell into a specific transmittance value.

In between the exit slit and the photocell is a chamber where samples may be placed. A clear specimen will yield 100% transmittance, while a turbid sample will deflect a considerable portion of the light rays and will have a lower percent transmittance. The greater the density, the lower the percent transmittance.

The wavelength selection is important and depends on the color of the suspension medium. However, it should not be changed during the experiment. It is customary to use 420 nm wavelength if the blank is nearly colorless, and 550 nm if it is yellowish.

When light is transmitted through a solution, some of it may be absorbed. If the absorption occurs in the ultraviolet or infrared regions of the electromagnetic spectrum, the solution will appear colorless. But if the absorption occurs in the visible region of the spectrum, the solution will appear colored. To give a simple example, a solution has a blue color when viewed by a transmitted white light (light containing all visible wavelengths and perhaps some UV and IR) because it absorbs a greater proportion of the nonblue wavelengths and transmits most of the blue wavelengths to one's eyes. Thus, white light entering this solution will emerge diminished in intensity and consist of a preponderance of blue wavelengths.

Quantitative photometric measurement of the absorption process is based on two observations, Lambert's Law and Beer's Law.

Lambert's Law

Lambert was working on the transmission of monochromatic light (light composed of only one wavelength) by homogeneous solid substances. He observed that the thickness of the solid material played a major role in the amount of light being transmitted through it; i.e., each successive unit layer of the medium absorbed an equal fraction of the light passing through it (Figure 1). The thickness of the medium is called the "light path length".

For example, suppose that the intensity of incident light upon some medium is 1.0 and that the medium is a colored plastic cube 1.0 cm thick, whose properties are such that the intensity of light emerging from the other side of the cube is diminished to 0.9. Now, if we add a second layer of the cube also 1.0 cm thick in the light path, the intensity of the light after passing

through the second cube layer will be 0.81 (0.9×0.9). If a third such cube is added, the intensity diminishes to 0.73 (0.9×0.81).

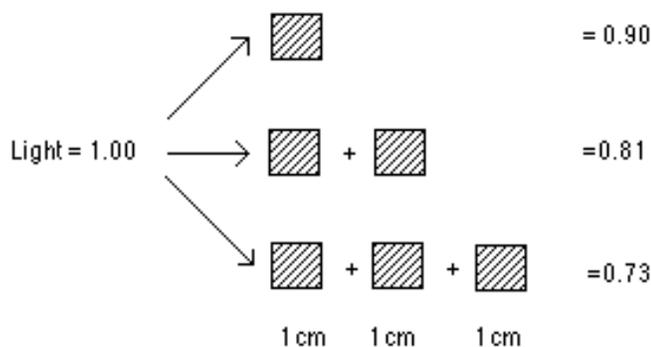


Fig. 1. Absorption of light passing through a series of colored cubes.

What this means is that the intensity of light transmitted is not linearly proportional to the thickness of the medium. If L is the thickness of the solid (or the length of the path of light through the medium) and T is the amount of light transmitted, the relationship between T and L will in general be as in Figure 2.

If the intensity of incident light is denoted by I_0 and that of transmitted light by I , then $\log_{10}(I_0/I)$ is called the optical density (O.D.) or absorbance (A) of the medium.

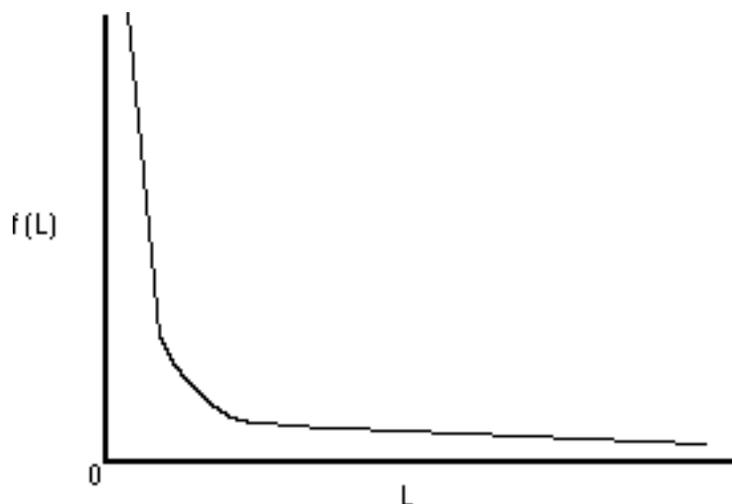


Fig. 2. The relationship between the transmitted light (T) and light path length (L).

Beer's Law

When Beer applied Lambert's Law to solutions, he found that both the concentration as well as the length of the light path affected light transmission through the solution. He demonstrated that the intensity obtained when light passed through a solution of concentration C and length L was equal to that obtained when that light passed through a solution of the same substance at concentration $2C$ and length $L/2$. Beer's Law is thus a generalized Lambert's Law

and simply states that light absorbance is proportional to the number of molecules of absorbing substance through which the light passes. In Beer's Law, O.D. takes into account the concentration of the solution, the length of the light path, as well as the wavelength of the incident light.

O.D. can be read directly off a spectrophotometer. At a fixed wavelength and fixed light path length, O.D. is proportional to the concentration of the light-absorbing molecules; it is this fact that allows us to use the spectrophotometer so advantageously in experiments. Since O.D. is a ratio of two numbers having the same dimensions, it is obviously a dimensionless quantity.

The fixed wavelength at which absorption measurements are made upon a given molecule is not arbitrary. It is customary to make such measurements at the wavelength (λ) of maximum absorption, the so-called λ_{max} of the molecule. The value of λ_{max} for a given molecule is determined experimentally by taking an absorption spectrum; i.e., the O.D. of a solution of the molecule (at a fixed concentration) is measured at successive closely-spaced wavelengths, and these O.D. values are plotted as a function of wavelength. The λ_{max} is immediately evident from such a graph.

There are two advantages in using λ_{max} to make experimental absorption measurements of a molecule: (1) λ_{max} is the wavelength at which there is the greatest change in O.D. per unit change in concentration (the experimental variable we are inevitably interested in if we are using O.D.) and (2) since the slope of the absorption spectrum is zero at λ_{max} only small errors in measurement of O.D. will be introduced if the wavelength dial of the spectrophotometer is slightly inaccurate or set slightly inaccurately.

Absorbance and Transmittance

Transmittance is simply the percentage of light impinging on a solution that passes through the solution and emerges to be detected by the instrument. It is zero for a completely opaque solution and 100% when all the light is transmitted. Transmittance and absorbance measure the same quantity but the scales are reversed and they are divided differently. Quite simply:

$$O.D. = A = \log_{10} \frac{1}{T}$$

For example, if the transmittance value for a solution is 42% (i.e. $T=0.42$), then its O.D. or A value would be $\log_{10} (1/0.42) = 0.38$.

The object in using the spectrophotometer is to measure the O.D. due to the presence of a specific molecule in a solution. But this molecule may be mixed with other molecules which also absorb light at the same wavelength. To negate such contributions to absorbance, the machine is arbitrarily set to read O.D. = 0.0 while actually measuring a solution called a "blank". The blank is a precise duplicate of the sample solution in components and concentrations except that the molecule whose absorbance is being examined is omitted. If the sample solution is measured next, an O.D. reading greater than zero is attributable only to that one extra absorbing solute.

In order that light absorption due only to that one solute be measured, the containers holding the blank and the sample must also have identical light absorption properties. The containers used to hold solutions for measurement in the spectrophotometer are special test

tubes (called cuvettes) produced in such a way as to have identical absorption characteristics. Cuvettes should always be placed in the machine in the same orientation. They should also be cleaned of fingerprints and water droplets with soft laboratory tissues before insertion into the machine.

A spectrophotometer contains the following:

- A light source (visible, UV, or both)
- A monochromator (to obtain a single fixed wavelength)
- A sample compartment (which holds a sample tube or cuvette of fixed path length)
- A photomultiplier (to magnify emergent light because its intensity is too diminished to be accurately read)
- And a phototube (to measure this light and display a reading on the meter panel; i.e., adjusts for the magnification relative to the original light source and gives accurate O.D. readings).

Operation of the Spectrophotometer

1. Turn on the instrument by rotating the Power Switch/Zero Control knob clockwise (see Fig. 3). This should be done 10 min before the measurements are to be made to give the machine enough time to warm up.
2. Select the proper wavelength. Turn the Wavelength Control knob, which is on top of the instrument, so that it registers the wavelength at which you want to measure your sample.
3. Adjust meter needle to "0" (left margin) by turning the Zero Control knob on the left.

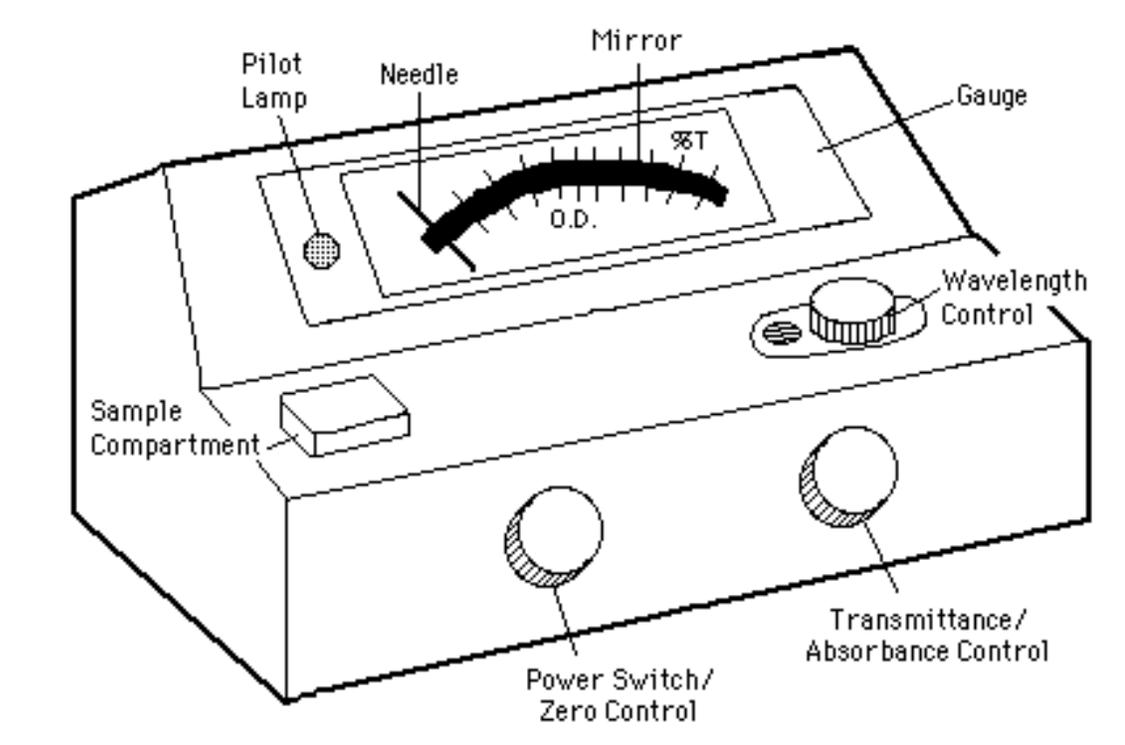


Fig. 3. A spectrophotometer.

4. Insert a cuvette containing 5 ml of control blank into the Sample Compartment. Adjust the meter needle to 100% transmittance by turning the Transmittance/ Absorbance Control knob (on the right). This knob regulates the amount of light passing through the exit slit. Now that the meter is adjusted for zero and 100 % transmittance, turbidity measurements can be made.
5. Remove the blank and insert the sample to be read and note the O.D. (bottom scale) or percent transmittance (top scale). Continue to read any other samples you may have. If you have a large number of samples to measure, recheck and adjust the transmittance using your blank after every ten readings. When you have finished your measurements, turn off the spectrophotometer, remove your blank or sample and clean the instrument.

Use of any section of this Lab Manual without the written consent of Dr. Eby Bassiri, Dept. of Biology, University of Pennsylvania is strictly prohibited.