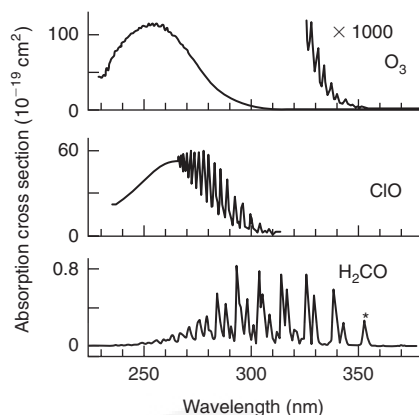
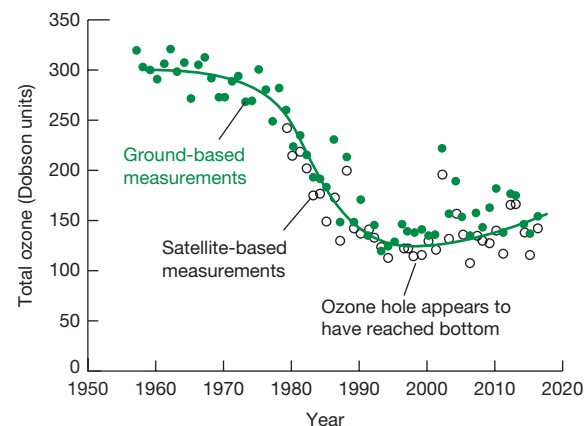


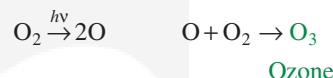
THE OZONE HOLE<sup>1</sup>

Spectra of trace gases in the atmosphere. At ozone's maximum absorption at a wavelength near 260 nm, a layer of ozone is more opaque than a layer of gold of the same mass. [Data from U. Platt and J. Stutz, *Differential Absorption Spectroscopy* (Berlin Heidelberg: Springer-Verlag, 2008).]



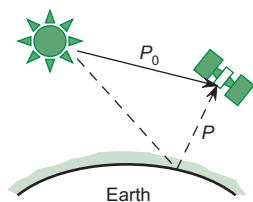
Spectroscopically measured atmospheric O<sub>3</sub> concentration at Halley on Bay Observatory Antarctica in October measured from the ground and with satellites. Dobson units are defined in Problem 18-15. [Data from NASA Ozone Watch, <https://ozonewatch.gsfc.nasa.gov/facts/history.html>]

Ozone, formed at altitudes of 20 to 40 km by the action of solar ultraviolet radiation ( $h\nu$ ) on O<sub>2</sub>, absorbs ultraviolet radiation that otherwise causes sunburn and skin cancer.



In 1985, the British Antarctic Survey reported that ozone in the Antarctic stratosphere had decreased by 50% in early spring (October), relative to levels observed in the preceding 20 years. Ground, airborne, and satellite-based observations have since shown that this “ozone hole” occurs only in early spring (Figure 1-1) and continued to form each year. Spectroscopy provided detailed understanding of the concentration and location of ozone and other trace gases in the atmosphere. These observations prompted research that demonstrated that chlorofluorocarbons, formerly used as propellant in spray cans and as a refrigerant, catalyze ozone decomposition. To protect life from ultraviolet radiation, the Montreal Protocol treaty negotiated in 1987 phased out chlorofluorocarbons. Safer substitutes such as difluoromethane and 1,2-difluoroethane are now in use, and ozone levels are beginning to rebound.

The Aura satellite, part of the convoy of five satellites known as the A-Train, orbits 705 km above Earth and circles the planet 14 times a day.<sup>2</sup> Its *differential optical absorption spectrometer* compares the intensity of solar irradiation ( $P_0$ ) from 270 to 500 nm with the radiance reflected by Earth ( $P$ ). Trace gases within the atmosphere absorb characteristic wavelengths of light, allowing identification and quantitation of O<sub>3</sub>, ClO, NO<sub>2</sub>, SO<sub>2</sub>, H<sub>2</sub>CO (formaldehyde), BrO, and OCIO. A 2-dimensional charge coupled device detector (Section 20-3), such as the camera in your smartphone, collects a “picture” of the atmosphere every 2 seconds with a spectral resolution of 0.5 nm, allowing it to monitor the narrow absorbance characteristics of many trace gases.



Satellite-borne differential optical absorption spectrometer. [Data from U. Platt and J. Stutz, *Differential Absorption Spectroscopy* (Berlin Heidelberg: Springer-Verlag, 2008).]



[C. Calvin/UCAR/NOAA]

Following the discovery of the Antarctic ozone “hole” in 1985, atmospheric chemist Susan Solomon led the first expedition in 1986 specifically intended to make chemical measurements of the Antarctic atmosphere using balloons and ground-based spectroscopy. The expedition discovered that ozone depletion occurred after

polar sunrise and that the concentration of chemically active chlorine in the stratosphere was ~100 times greater than had been predicted from gas-phase chemistry. Solomon’s group identified chlorine as the culprit in ozone destruction and polar stratospheric clouds as the catalytic surface responsible for the release of so much chlorine.

**Spectrophotometry** is any technique that uses light to measure chemical concentrations. A procedure based on absorption of visible light is called *colorimetry*. Chapter 18 is intended to give a stand-alone overview of spectrophotometry sufficient for introductory purposes. Chapter 19 goes further into applications, and Chapter 20 discusses instrumentation.

## 18-1 Properties of Light

It is convenient to describe light in terms of both particles and waves. Light waves consist of perpendicular, oscillating electric and magnetic fields. For simplicity, a *plane-polarized* wave is shown in Figure 18-1. In this figure, the electric field is in the *xy*-plane, and the magnetic field is in the *xz*-plane. **Wavelength**,  $\lambda$ , is the crest-to-crest distance between waves. **Frequency**,  $\nu$ , is the number of complete oscillations that the wave makes each second. The unit of frequency is 1/second. One oscillation per second is called one **hertz** (Hz). A frequency of  $10^6 \text{ s}^{-1}$  is  $10^6$  Hz, or 1 *megahertz* (MHz). Ordinary, *unpolarized* light has electromagnetic components in all planes parallel to the direction of travel.

The relation between frequency and wavelength is

$$\text{Relation between frequency and wavelength: } \nu\lambda = c \quad (18-1)$$

where  $c$  is the speed of light ( $2.998 \times 10^8 \text{ m/s}$  in vacuum). In a medium other than vacuum, the speed of light is  $c/n$ , where  $n$  is the **refractive index** of that medium. For visible wavelengths in most substances,  $n > 1$ , so visible light travels more slowly through matter than through vacuum. When light moves between media with different refractive indices, the frequency remains constant but the wavelength changes.

With regard to energy, it is more convenient to think of light as particles called **photons**. Each photon carries energy  $E$  given by

$$\text{Relation between energy and frequency: } E = h\nu \quad (18-2)$$

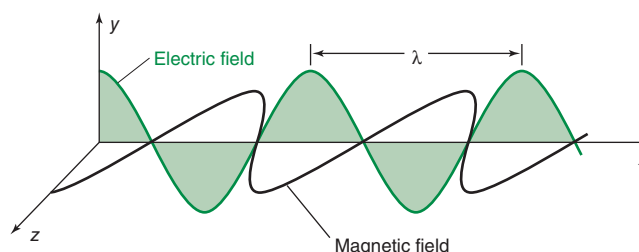
where  $h$  is *Planck’s constant* ( $= 6.626 \times 10^{-34} \text{ J}\cdot\text{s}$ ).

Equation 18-2 states that energy is proportional to frequency. Combining Equations 18-1 and 18-2, we can write

$$E = \frac{hc}{\lambda} = hc\tilde{\nu} \quad (18-3)$$

where  $\tilde{\nu} (= 1/\lambda)$  is called **wavenumber**. Energy is inversely proportional to wavelength and directly proportional to wavenumber. Red light, with a longer wavelength than blue light, is less energetic than blue light. The most common unit of wavenumber is  $\text{cm}^{-1}$ , read “reciprocal centimeters” or “wavenumbers.”

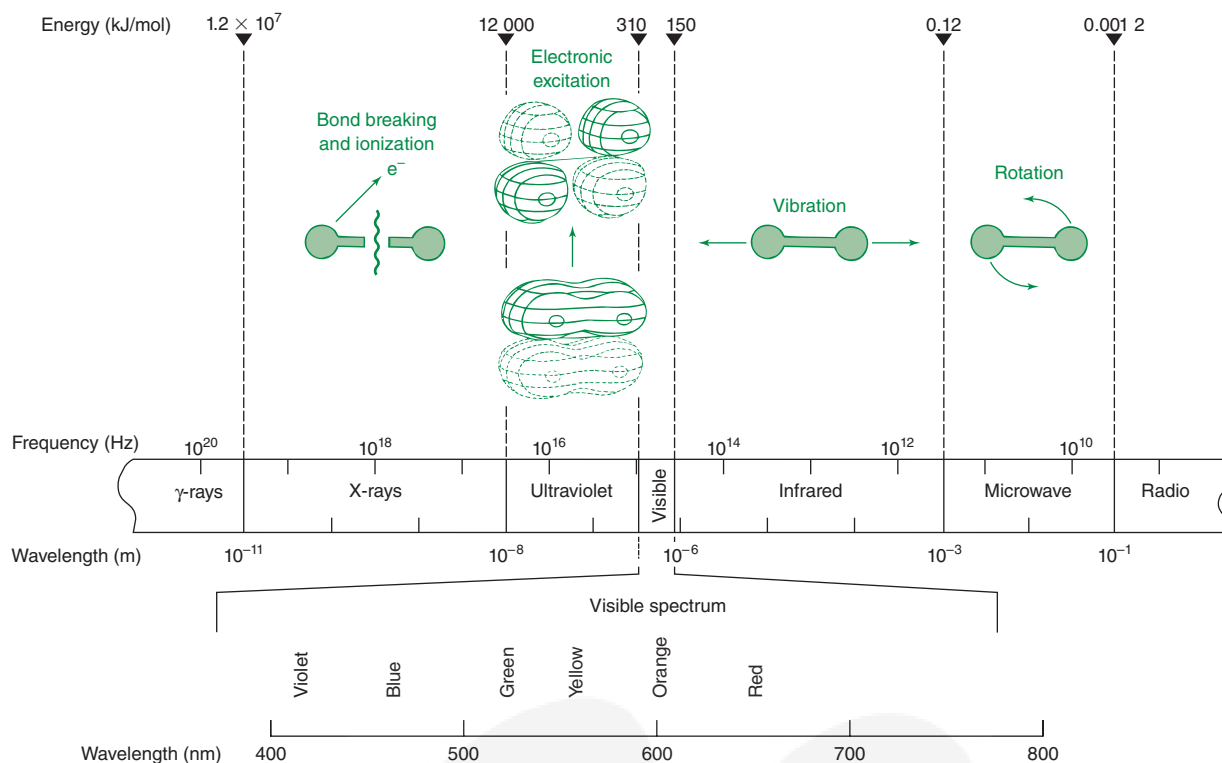
The names of regions of the **electromagnetic spectrum** in Figure 18-2 are historical. There are no abrupt changes in characteristics as we go from one region to the next, such as visible to infrared. Visible light—the kind of electromagnetic radiation we see—represents only a small fraction of the electromagnetic spectrum. Wave properties govern light behavior such as interference and diffraction. Interaction with chemicals is described using the particle nature of light—the photon and its energy.



**FIGURE 18-1** Plane-polarized electromagnetic radiation of wavelength  $\lambda$ , propagating along the *x*-axis. The electric field of plane-polarized light is confined to a single plane.

$E \equiv$  energy  
 $\nu \equiv$  frequency  
 $E \propto \nu$   
 $\lambda \equiv$  wavelength  
 $E \propto 1/\lambda$

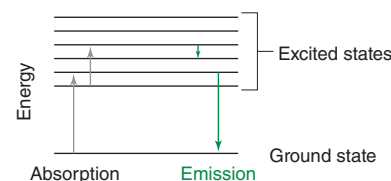
**Wave-particle duality of light:** Light behaves as a wave with a wavelength and frequency and exhibits the wave phenomena of interference, diffraction, and reflection. Light also behaves as a particle (a photon) that carries a discrete energy that can be absorbed or emitted by a molecule.



**FIGURE 18-2** Electromagnetic spectrum, showing representative molecular processes that occur when light in each region is absorbed. Visible light spans the wavelength range 380–780 nanometers ( $1 \text{ nm} = 10^{-9} \text{ m}$ ).

## 18-2 Absorption of Light

When a molecule absorbs a photon, the energy of the molecule increases. We say that the molecule is promoted to an **excited state** (Figure 18-3). If a molecule emits a photon, the energy of the molecule is lowered. The lowest energy state of a molecule is called the **ground state**. Figure 18-2 indicates that microwave radiation stimulates rotation of molecules when it is absorbed. Infrared radiation stimulates vibrations. Visible and ultraviolet radiation promote electrons to higher energy orbitals. Short-wavelength ultraviolet and X-ray radiation are harmful because they break chemical bonds and ionize molecules, which is why you should minimize your exposure to direct sunlight and medical X-rays.



**FIGURE 18-3** Absorption of light increases the energy of a molecule. Emission of light decreases its energy.

### EXAMPLE Photon Energies

By how many kilojoules per mole is the energy of  $\text{O}_2$  increased when it absorbs ultraviolet radiation with a wavelength of 147 nm? How much is the energy of  $\text{CO}_2$  increased when it absorbs infrared radiation with a wavenumber of  $2300 \text{ cm}^{-1}$ ?

**Solution** For the ultraviolet radiation, the energy increase is

$$\begin{aligned}\Delta E &= h\nu = h \frac{c}{\lambda} \\ &= (6.626 \times 10^{-34} \text{ J} \cdot \text{s}) \left[ \frac{(2.998 \times 10^8 \text{ m/s})}{(147 \text{ nm})(10^{-9} \text{ m/nm})} \right] = 1.35 \times 10^{-18} \text{ J/molecule} \\ (1.35 \times 10^{-18} \text{ J/molecule})(6.022 \times 10^{23} \text{ molecules/mol}) &= 814 \text{ kJ/mol}\end{aligned}$$

This is enough energy to break the  $\text{O}=\text{O}$  bond in oxygen. For  $\text{CO}_2$ , the energy increase is

$$\begin{aligned}\Delta E &= h\nu = h \frac{c}{\lambda} = hc\tilde{\nu} \quad \left( \text{recall that } \tilde{\nu} = \frac{1}{\lambda} \right) \\ &= (6.626 \times 10^{-34} \text{ J} \cdot \text{s})(2.998 \times 10^8 \text{ m/s})(2300 \text{ cm}^{-1})(100 \text{ cm/m}) \\ &= 4.6 \times 10^{-20} \text{ J/molecule} = 28 \text{ kJ/mol}\end{aligned}$$

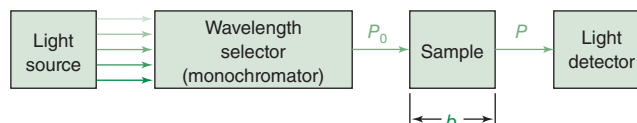
Infrared absorption increases the amplitude of the vibrations of the CO<sub>2</sub> bonds.

**TEST YOURSELF** What is the wavelength, wavenumber, and name of radiation with an energy of 100 kJ/mol? (**Answer:** 1.20 μm, 8.36 × 10<sup>3</sup> cm<sup>-1</sup>, infrared)

**Irradiance** is the energy per unit time per unit area in the light beam (watts per square meter, W/m<sup>2</sup>). The terms “intensity” or “radiant power” have been used for this same quantity.

**Monochromatic light** consists of “one color” (one wavelength). The better the monochromator, the narrower is the range of wavelengths in the emerging beam.

When light is absorbed by a sample, the *irradiance* of the beam of light is decreased. **Irradiance**,  $P$ , is the energy per second per unit area of the light beam. A rudimentary spectrophotometric experiment is illustrated in Figure 18-4. Light passes through a **monochromator** (a prism, a grating, or a filter) to select one wavelength (Color Plate 14). Light with a narrow range of wavelengths is said to be **monochromatic** (“one color”). The monochromatic light, with irradiance  $P_0$ , travels through a sample along a path of length  $b$  within the sample. The irradiance of the beam emerging from the other side of the sample is  $P$ . Some of the light may be absorbed by the sample, so  $P \leq P_0$ .



**FIGURE 18-4** Schematic diagram of a single-beam spectrophotometer.  $P_0$ , irradiance of beam entering sample;  $P$ , irradiance of beam emerging from sample;  $b$ , length of path through sample. Operation and use of a single-beam spectrophotometer are demonstrated in the video at [www.youtube.com/watch?v=xHQM4BbR040](http://www.youtube.com/watch?v=xHQM4BbR040).

**Transmittance**,  $T$ , is defined as the fraction of the original light that passes through the sample.

Transmittance:

$$T = \frac{P}{P_0} \quad (18-4)$$

Therefore,  $T$  has the range 0 to 1. The *percent transmittance* ( $= 100T$ ) ranges between 0 and 100%. **Absorbance** is defined as

Absorbance:

$$A = \log\left(\frac{P_0}{P}\right) = -\log T \quad (18-5)$$

When no light is absorbed,  $P = P_0$  and  $A = 0$ . If 90% of the light is absorbed, 10% is transmitted and  $P = 0.10P_0$ . This ratio gives  $A = 1$ . If only 1% of the light is transmitted,  $A = 2$ . Absorbance used to be called *optical density*.

Absorbance is so important because it is directly proportional to the concentration,  $c$ , of the light-absorbing species in the sample (Color Plate 15).

Beer's law:

$$A = \epsilon bc \quad (18-6)$$

Equation 18-6, which is the heart of spectrophotometry as applied to analytical chemistry, is called the *Beer-Lambert law*,<sup>3</sup> or simply **Beer's law**. Absorbance is dimensionless, but some people write “absorbance units” after absorbance. The concentration of the sample,  $c$ , is usually given in units of moles per liter (M). The pathlength,  $b$ , is commonly expressed in centimeters. The quantity  $\epsilon$  (epsilon) is called the **molar absorptivity** (or *extinction coefficient* in older literature) and has units M<sup>-1</sup> cm<sup>-1</sup> to make the product  $\epsilon bc$  dimensionless. Molar absorptivity tells how much light is absorbed at a particular wavelength per cm of path through a 1 M solution of a particular substance.

### EXAMPLE Absorbance, Transmittance, and Beer's Law

Find the absorbance and transmittance of a 0.002 40 M solution of a substance with a molar absorptivity of 313 M<sup>-1</sup> cm<sup>-1</sup> in a cell with a 2.00-cm pathlength.

**Solution** Equation 18-6 gives us the absorbance.

$$A = \epsilon bc = (313 \text{ M}^{-1} \text{ cm}^{-1})(2.00 \text{ cm})(0.002 40 \text{ M}) = 1.50$$

Relation between transmittance and absorbance:

$P/P_0$	% $T$	$A$
1	100	0
0.1	10	1
0.01	1	2

Box 18-1 explains why absorbance, not transmittance, is directly proportional to concentration.

Transmittance is obtained from Equation 18-5 by raising 10 to the power equal to the expression on each side of the equation:

$$\log T = -A$$

$$T = 10^{\log T} = 10^{-A} = 10^{-1.50} = 0.0316$$

If  $x = y$ ,  $10^x = 10^y$ .

Just 3.2% of the incident light emerges from this solution.

**TEST YOURSELF** The transmittance of a 0.010 M solution of a compound in a 0.100-cm pathlength cell is  $T = 8.23\%$ . Find the absorbance ( $A$ ) and the molar absorptivity ( $\epsilon$ ) of the compound. (**Answer:** 1.08,  $1.08 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$ )

Equation 18-6 could be written

$$A_\lambda = \epsilon_\lambda bc$$

because  $A$  and  $\epsilon$  depend on the wavelength of light. The quantity  $\epsilon$  is a coefficient of proportionality between absorbance and the product  $bc$ . The greater the molar absorptivity, the greater the absorbance. An **absorption spectrum** (Demonstration 18-1) is a graph showing how  $A$  (or  $\epsilon$ ) varies with wavelength. Spectra of hundreds of compounds are available online.<sup>4</sup>

The part of a molecule responsible for light absorption is called a **chromophore**. Any substance that absorbs visible light appears colored when white light is transmitted through it or reflected from it. White light contains all the colors in the visible spectrum. The substance absorbs certain wavelengths of the white light, and our eyes detect the wavelengths that are not absorbed. Table 18-1 gives a rough guide to colors.<sup>5</sup> The observed color is called the *complement* of the absorbed color. For example, bromophenol blue has maximum absorbance at 591 nm (orange) and its observed color is blue. Color Plates 16 and 17 display absorption spectra and observed colors.

The plural of "spectrum" is "spectra."

The color of a solution is the **complement** of the color of the light that it absorbs. The color we perceive depends not only on the wavelength of light, but also on its intensity.

### BOX 18-1 Why Is There a Logarithmic Relation Between Transmittance and Concentration?<sup>6</sup>

Beer's law, Equation 18-6, states that *absorbance* is proportional to the concentration of the absorbing species. The fraction of light passing through a sample (the *transmittance*) is related logarithmically, not linearly, to the sample concentration. Why should this be?

Imagine light of irradiance  $P$  passing through an *infinitesimally thin* layer of solution whose thickness is  $dx$ . A physical model of the absorption process suggests that, within the infinitesimally thin layer, decrease in power ( $dP$ ) ought to be proportional to the incident power ( $P$ ), to the concentration of absorbing species ( $c$ ), and to the thickness of the section ( $dx$ ):

$$dP = -\beta Pc \, dx \quad (\text{A})$$

where  $\beta$  is a constant of proportionality and the minus sign indicates a decrease in  $P$  as  $x$  increases. The rationale for saying that the decrease in power is proportional to the incident power may be understood from a numerical example. If 1 photon out of 1 000 incident photons is absorbed in a thin layer, we would expect that 2 out of 2 000 incident photons would be absorbed. The decrease in photons (power) is proportional to the incident flux of photons (power).

Equation A can be rearranged and integrated to find an expression for  $P$ :

$$-\frac{dP}{P} = \beta c \, dx \Rightarrow -\int_{P_0}^P \frac{dP}{P} = \beta c \int_0^b dx$$

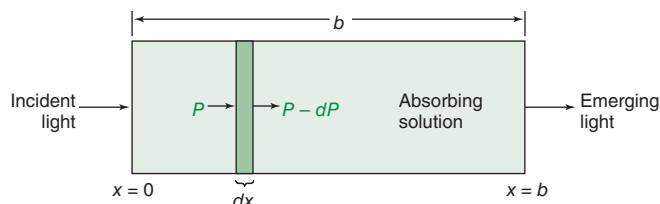
The limits of integration are  $P = P_0$  at  $x = 0$  and  $P = P$  at  $x = b$ . The integral on the left side is  $\int dP/P = \ln P$ , so

$$-\ln P - (-\ln P_0) = \beta cb \Rightarrow \ln\left(\frac{P_0}{P}\right) = \beta cb$$

Finally, converting  $\ln$  to  $\log$ , using the relation  $\ln z = (\ln 10)(\log z)$ , gives Beer's law:

$$\underbrace{\log\left(\frac{P_0}{P}\right)}_{\text{Absorbance}} = \underbrace{\left(\frac{\beta}{\ln 10}\right)}_{\text{Constant} \equiv \epsilon} cb \Rightarrow A = \epsilon cb$$

The logarithmic relation of  $P_0/P$  to concentration arises because in each infinitesimal portion of the total volume, *the decrease in power is proportional to the power incident upon that section*. As light travels through a sample, power loss in each succeeding layer decreases because less incident power reaches that layer. Molar absorptivity ranges from 0 (if the probability for photon absorption is 0) to approximately  $10^5 \text{ M}^{-1} \text{ cm}^{-1}$  (when the probability for photon absorption approaches unity).





## DEMONSTRATION 18-1 Absorption Spectra

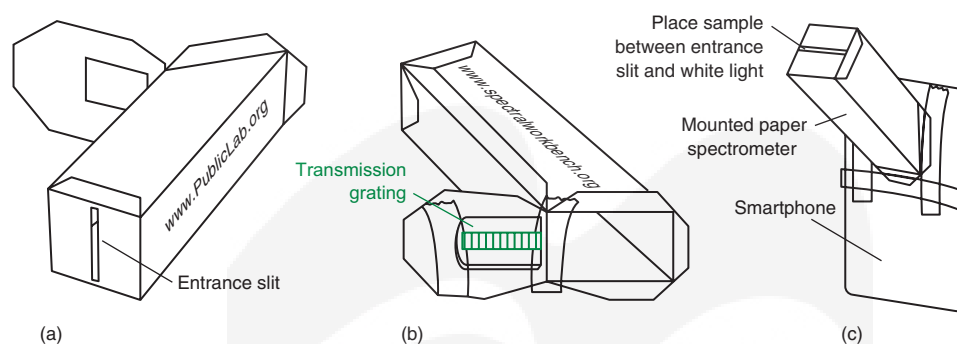
A simple (and delicious) demonstration of the relation between observed and absorbed color requires gummy bear candy, a penlight or white light-emitting diode (Box 18-2), a red laser pointer, and a green laser pointer.<sup>7</sup> Place a red and a green gummy bear on plain white paper. In a darkened room place the white light source against each gummy bear. Observe the transmitted color, which is the complement of the absorbed color (Table 18-1). Confirm this by shining the red laser pointer on the red gummy bear and on the green gummy bear. Repeat with the green laser pointer.

To investigate the effect of pathlength on absorbance, shine the red laser pointer through the length of the green gummy. Note how the intensity fades as the distance traveled through the bear (pathlength) increases. Complementary colors can also be demonstrated using colored light-emitting diodes.<sup>8</sup>

The spectrum of visible light can be observed using a papercraft or a 3D-printed smartphone spectrometer.<sup>9</sup> When viewing a white

light source, the full visible spectrum is observed. By placing a beaker of colored solution between the entrance slit of the spectrometer and the white light, you see the transmitted colors. The spectrum loses intensity at wavelengths absorbed by the colored species.

Color Plate 16a shows the spectrum of white light and the spectra of four different colored solutions. Potassium dichromate, which appears orange or yellow, absorbs blue wavelengths. Bromophenol blue absorbs orange and appears blue to our eyes. Phenolphthalein and rhodamine 6G absorb in the center of the visible spectrum. For comparison, spectra of these four solutions recorded with a spectrophotometer are shown in Color Plate 16b. This same setup can be used to demonstrate fluorescence of Rhodamine 6G using a green laser pointer as shown in Color Plate 16c. Other experiments and demonstrations have been developed for portable and smartphone spectrometers.<sup>10</sup>



Foldable paper spectrometer (a) front view (b) back view with transmission grating made from DVD disk (c) mounted on smartphone [Information from [www.PublicLab.org](http://www.PublicLab.org)]

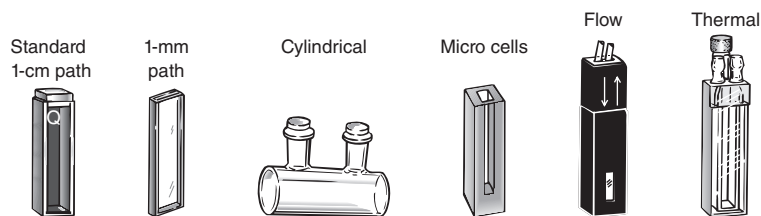
TABLE 18-1 Colors of visible light

Wavelength of maximum absorption (nm)	Color absorbed	Color observed
380–420	Violet	Green-yellow
420–440	Violet-blue	Yellow
440–470	Blue	Orange
470–500	Blue-green	Red
500–520	Green	Purple-red
520–550	Yellow-green	Violet
550–580	Yellow	Violet-blue
580–620	Orange	Blue
620–680	Red-orange	Blue-green
680–780	Red	Green

### 18-3 Measuring Absorbance

The minimum requirements for a spectrophotometer (a device to measure absorbance of light) were shown in Figure 18-4. Light from a continuum-wavelength source passes through a monochromator, which selects a narrow band of wavelengths from the incident beam. This “monochromatic” light travels through a sample of pathlength  $b$ , and the irradiance of the emergent light is measured.

For visible and ultraviolet spectroscopy, a liquid sample is usually contained in a cell called a **cuvette** that has flat, fused-silica ( $\text{SiO}_2$ ) faces (Figure 18-5). Glass is suitable for



**FIGURE 18-5** Common cuvettes for visible and ultraviolet spectroscopy. Micro cells use smaller volumes of solution. Flow cells permit continuous flow of solution through the cell. In the thermal cell, liquid from a constant-temperature bath flows through the cell jacket to maintain a desired temperature. [Information from A. H. Thomas Co., Philadelphia, PA.]

visible spectroscopy, but not ultraviolet because it absorbs ultraviolet radiation. “UV” or “Q” at the top of the cuvette identifies cells suitable for the ultraviolet. The most common cuvettes have a 1.000-cm pathlength and are sold in matched sets for sample and reference. Standard cells hold 2.5 to 4.5 mL. Micro cells use sample volumes down to 70  $\mu\text{L}$ . Polystyrene and methacrylate are suitable for visible spectroscopy but are not compatible with organic solvents. Cyclic olefin copolymer may be used in ultraviolet and visible spectroscopy and has greater solvent compatibility.

For infrared measurements, cell materials are selected based on their infrared transparency and compatibility with solvent. Cells are commonly constructed of NaCl or KBr, but these are sensitive to moisture. KRS-5 (TlBr-TlI), ZnSe, and diamond are insoluble in water. For the 600 to 10  $\text{cm}^{-1}$  far-infrared region, polyethylene and diamond are transparent windows. Liquid samples are prepared in solvents such as carbon disulfide, carbon tetrachloride, and dichloromethane with weak infrared absorbance. Water and alcohols absorb strongly in the infrared and are not compatible with many cell materials. Pathlengths are short (0.1–1 mm) to minimize background absorbance due to solvent. Adjustable cells use replaceable spacers between the cell windows to set the pathlength, while fixed cells have a predetermined pathlength (Figure 20-34). Solid samples are commonly ground to a fine powder, which can be added to mineral oil (a viscous hydrocarbon also called Nujol) to give a dispersion that is called a *mull* and is pressed between two KBr plates.<sup>11</sup> The analyte spectrum is obscured in a few regions in which the mineral oil absorbs infrared radiation. Alternatively, a 1 wt% mixture of solid sample with KBr can be ground to a fine powder and pressed into a translucent pellet at a pressure of  $\sim 60$  MPa (600 bar). Mulls and KBr are predominantly used for qualitative analysis, as pathlengths and concentrations are difficult to reproduce.

Gases are more dilute than liquids and require cells with longer pathlengths, typically ranging from 10 cm to many meters. A pathlength of many meters is obtained in an instrument by reflecting light so that it traverses the sample many times before reaching the detector. Atmospheric measurements with pathlengths of kilometers require *collimated* (beam of parallel rays) light sources such as lasers (Section 20-1).

Paper, solids, and powders can also be examined by *diffuse reflectance*, in which reflected radiation, instead of transmitted radiation, is observed. Wavelengths absorbed by the sample are not reflected as well. This technique is sensitive only to the surface of the sample.

Figure 18-4 outlines a *single-beam* instrument, which has only one light path. We do not measure the incident irradiance,  $P_0$ , directly. Figure 18-6 shows some irradiance is lost to scattering and reflection. Dust and air bubbles scatter light, causing random change in transmitted irradiance. Reflection depends on the refractive index of the cuvette and sample, and on positioning of the cuvette. To correct for these, the irradiance of light passing through a reference cuvette containing pure solvent (or a reagent blank) is defined as  $P_0$ . This cuvette is then removed and replaced by an identical one containing sample. The irradiance of light striking the detector after passing through the sample is the quantity  $P$ . Knowing both  $P$  and  $P_0$  allows  $T$  and  $A$  to be determined. The reference cuvette compensates for reflection and absorption by the cuvette and solvent. The reference cuvette containing pure solvent acts as a blank in the measurement of transmittance. A *double-beam* instrument, which splits the light to pass alternately between sample and reference cuvettes, is shown in Figures 20-1, 20-2, and 20-3.

In recording an absorbance spectrum, first record a *baseline spectrum* with reference solutions (pure solvent or a reagent blank) in both the sample and reference cuvettes. Cuvettes are sold in matched sets that are as identical as possible to each other. If the cuvettes and the instrument were perfect, the baseline would be 0 everywhere. In our imperfect world,

#### Useful wavelength range and cost of cuvettes:

Glass	340–2 500 nm	\$\$
Quartz	190–2 500 nm	\$\$\$

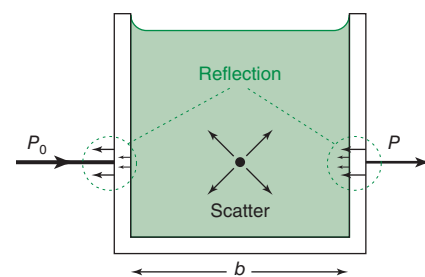
#### Disposable cuvettes:

Polystyrene	340–900 nm	¢
Methacrylate	300–900 nm	¢
Cyclic olefin copolymer	220–900 nm	¢¢

#### Useful range for common infrared windows:

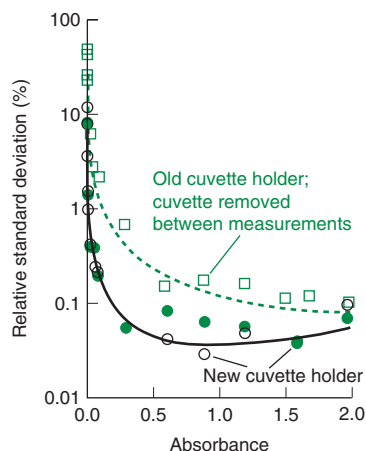
NaCl	28 000–700 $\text{cm}^{-1}$
KBr	33 000–400 $\text{cm}^{-1}$
CsI	33 000–150 $\text{cm}^{-1}$
KRS-5	16 000–200 $\text{cm}^{-1}$
ZnSe	20 000–500 $\text{cm}^{-1}$
Diamond	45 000–10 $\text{cm}^{-1}$
Sapphire ( $\text{Al}_2\text{O}_3$ )	66 000–2 000 $\text{cm}^{-1}$
Polyethylene	600–10 $\text{cm}^{-1}$

[Data from Bruker Optics.]



**FIGURE 18-6** Reflection and scatter, in addition to absorption, attenuate light passing through a cuvette.

An empty glass cuvette reflects 13% of incident visible irradiance. You have seen this reflectance from glass when you tried taking a flash picture through a window.



**FIGURE 18-7** Precision of replicate absorbance measurements with a diode array spectrometer at 350 nm with a dichromate solution. Filled circles are from replicate measurements in which the sample was not removed from the cuvette holder between measurements. Open circles are from measurements in which the sample was removed and then replaced in the cuvette holder between measurements. Best reproducibility is observed at intermediate absorbance ( $A \approx 0.3 - 2$ ). Note logarithmic ordinate. Lines are least-squares fit of data to theoretical equations. [Data from J. Galbán, S. de Marcos, I. Sanz, C. Ubide, and J. Zuriarrain, "Uncertainty in Modern Spectrophotometers," *Anal. Chem.* **2007**, 79, 4763.]

Don't touch the clear faces of a cuvette — fingerprints scatter and absorb light.

the baseline usually exhibits small positive and negative absorbance. Subtract the baseline absorbance from the sample absorbance to obtain the true absorbance at each wavelength.

For spectrophotometric analysis, we normally choose the wavelength of maximum absorbance for two reasons: (1) The sensitivity of the analysis is greatest at maximum absorbance; that is, we get the maximum response for a given concentration of analyte. (2) The absorption spectrum is relatively flat at the maximum, so there is little variation in absorbance if the monochromator drifts a little or if the width of the transmitted band changes slightly.

Typically, relative uncertainties are 1–3% for simple spectroscopic analyses, and often more for complex procedures or when the colored species is unstable. The primary sources of uncertainty are the reproducibility associated with sample preparation, the spectrophotometric reaction, and interferences,<sup>12</sup> but other factors can contribute to error.

Modern spectrophotometers are most precise (reproducible) at intermediate levels of absorbance ( $A \approx 0.3$  to 2). If too little light gets through the sample (high absorbance), it is hard to measure the small amount of transmitted light accurately. If too much light gets through (low absorbance), it is hard to distinguish transmittance of the sample from that of the reference. It is desirable to adjust sample concentration so that absorbance falls in this intermediate range.

Figure 18-7 shows the relative standard deviation of replicate measurements made at 350 nm with a diode array spectrometer. Filled circles are from replicate measurements in which the sample was not removed from the cuvette holder between measurements. Open circles are from measurements in which the sample was removed and then replaced in the cuvette holder between measurements. Relative standard deviation is below 0.1% in both cases for absorbance in the range 0.3 to 2. Data points represented by open squares were obtained when a 10-year-old cuvette holder was used and the sample was removed and replaced in the holder between measurements. Variability in the position of the cuvette more than doubles the relative standard deviation. The conclusion is that modern spectrometers with modern cell holders provide excellent reproducibility, while precision decreases with old cell holders and sample manipulation between measurements.

Always keep containers covered to exclude dust, which scatters light and appears to the spectrophotometer to be absorbance. Filtering the final solution through a very fine filter (for example, 0.5  $\mu\text{m}$ ) may be necessary in critical work. Monitor solutions with concentrated matrices such as blood serum for formation of precipitate after filtration. Handle cuvettes on the frosted sides and use a tissue to avoid putting fingerprints on the faces. Keep cuvettes scrupulously clean.

Slight mismatch between sample and reference cuvettes can lead to systematic errors in spectrophotometry. Place cuvettes in the spectrophotometer as reproducibly as possible. Variation in apparent absorbance arises from slightly misplacing the cuvette in its holder, turning a flat cuvette around by 180°, or rotating a circular cuvette.

## 18-4 Beer's Law in Chemical Analysis

Spectrophotometric analysis may be performed in four ways: (1) directly on an analyte if it absorbs at a unique wavelength, (2) after converting analyte to a product that absorbs at a unique wavelength, (3) on a mixture of analytes that possess different absorption spectra by measuring absorbance at multiple wavelengths (Section 19-1), and (4) by separating similarly absorbing analytes using a technique such as chromatography (Chapter 23) so that the absorbance of each analyte may be measured individually.

For a solution with multiple components, the absorbance at any wavelength is the sum of absorbances of all components at that wavelength. Most compounds absorb ultraviolet radiation, so measurements in this region of the spectrum are prone to interference by other compounds. Direct analysis is usually restricted to the visible spectrum. If there are no interfering species, however, ultraviolet absorbance is satisfactory. DNA and RNA (Appendix L) are assayed in the ultraviolet region because aromatic nucleotides absorb near 260 nm (see Problem 18-21).<sup>13</sup> Proteins are assayed at 280 nm because the aromatic amino acids tyrosine and tryptophan (Table 10-1) present in most proteins absorb in the ultraviolet (see Problem 18-22).<sup>14</sup>

### EXAMPLE Direct Measurement of Benzene in Hexane

(a) Pure hexane has negligible ultraviolet absorbance above a wavelength of 200 nm. A solution prepared by dissolving 25.8 mg of benzene ( $\text{C}_6\text{H}_6$ , FM 78.11) in hexane and diluting to 250.0 mL had an absorption peak at 256 nm with an absorbance of 0.266 in a 1.000-cm cell. Find the molar absorptivity of benzene at this wavelength.

This example illustrates the measurement of molar absorptivity from a single solution. It is better to measure several concentrations to obtain a more reliable absorptivity and to demonstrate that Beer's law is obeyed.



**Solution** The concentration of benzene is

$$[\text{C}_6\text{H}_6] = \frac{(0.0258 \text{ g})/(78.11 \text{ g/mol})}{0.2500 \text{ L}} = 1.321 \times 10^{-3} \text{ M}$$

We find the molar absorptivity from Beer's law:

$$\text{Molar absorptivity} = \epsilon = \frac{A}{bc} = \frac{(0.266)}{(1.000 \text{ cm})(1.321 \times 10^{-3} \text{ M})} = 201.4 \text{ M}^{-1} \text{ cm}^{-1}$$

(b) A sample of hexane contaminated with benzene had an absorbance of 0.070 at 256 nm in a cuvette with a 5.000-cm pathlength. Find the concentration of benzene in mg/L.

**Solution** Using Beer's law with the molar absorptivity from part (a), we find:

$$[\text{C}_6\text{H}_6] = \frac{A}{\epsilon b} = \frac{0.070}{(201.4 \text{ M}^{-1} \text{ cm}^{-1})(5.000 \text{ cm})} = 6.95 \times 10^{-5} \text{ M}$$
$$[\text{C}_6\text{H}_6] = \left(6.95 \times 10^{-5} \frac{\text{mol}}{\text{L}}\right) \left(78.11 \times 10^3 \frac{\text{mg}}{\text{mol}}\right) = 5.4 \frac{\text{mg}}{\text{L}}$$

**TEST YOURSELF** 0.10 mM  $\text{KMnO}_4$  has an absorbance maximum of 0.26 at 525 nm in a 1.000-cm cell. Find the molar absorptivity and the concentration of a solution whose absorbance is 0.52 at 525 nm in the same cell. (**Answer:**  $2600 \text{ M}^{-1} \text{ cm}^{-1}$ , 0.20 mM)

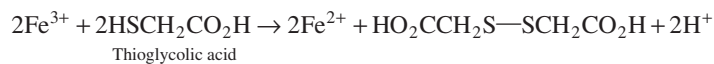
### Formation of an Absorbing Product: Serum Iron Determination

Spectrophotometric analysis can be performed by reacting analyte with reagents under prescribed conditions to form a colored product. The reaction should be one that goes to completion, so reagent concentrations might be tens or hundreds of times that of the analyte to force the reaction. The product should be stable. A wait time may be required to allow sufficient time for the reaction to go to completion, but not so long that the product decomposes. The reagents should ideally have zero absorbance at the monitored wavelength and should not form products with other species in the sample. Additional reagents (masking agents) may be needed to prevent other species from reacting. The determination of serum iron will illustrate these steps.

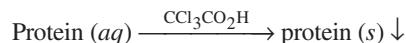
Iron for biosynthesis is transported through the bloodstream by the protein *transferrin*. The following procedure measures the Fe content of transferrin in blood.<sup>15</sup> This analysis requires about 1  $\mu\text{g}$  of Fe for an accuracy of 2–5%. Human blood usually contains about 45 vol% cells and 55 vol% *plasma* (liquid). If blood is collected without an anticoagulant, the blood clots; the liquid that remains is called *serum*. Serum normally contains about 1  $\mu\text{g}$  of Fe/mL attached to transferrin.

The serum iron determination has three steps:

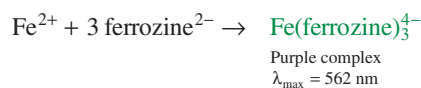
**Step 1** Reduce  $\text{Fe}^{3+}$  in transferrin to  $\text{Fe}^{2+}$ , which is released from the protein. Suitable reducing agents include thioglycolic acid, hydroxylamine hydrochloride ( $\text{NH}_3\text{OH}^+\text{Cl}^-$ ), and ascorbic acid.



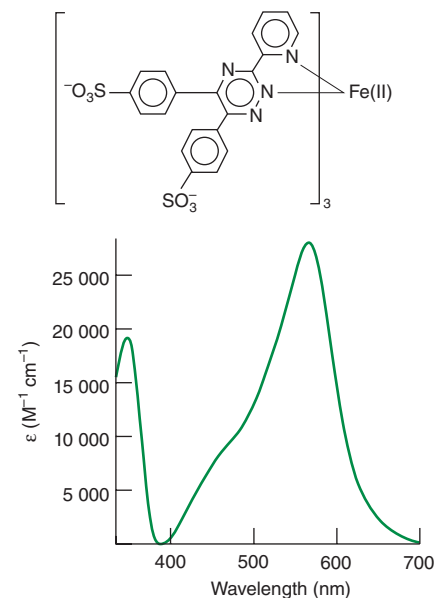
**Step 2** Add trichloroacetic acid ( $\text{CCl}_3\text{CO}_2\text{H}$ ) to precipitate proteins, leaving  $\text{Fe}^{2+}$  in solution. Centrifuge the mixture to remove the precipitate. If protein were left in the solution, it would partially precipitate in the final solution. Light scattered by the precipitate would be mistaken for absorbance.



**Step 3** Transfer a measured volume of supernatant liquid from step 2 to a fresh vessel. Add buffer plus excess ferrozine to form a purple complex. Measure the absorbance at the 562-nm peak (Figure 18-8). Buffer sets a pH at which complex formation is complete.

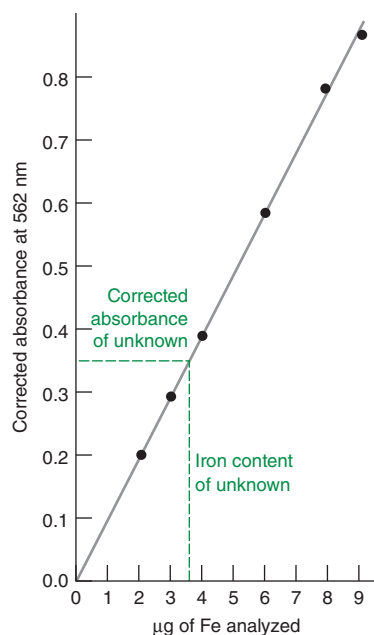


In most spectrophotometric analyses, it is important to prepare a **reagent blank** containing all reagents, but with analyte solution replaced by distilled water. Any absorbance of the blank



**FIGURE 18-8** Visible absorption spectrum of the complex  $\text{Fe(II)(ferrozine)}_3^{4-}$  used in the colorimetric analysis of serum iron.

**Supernate** is the liquid layer above the solid that collects at the bottom of a tube during centrifugation.



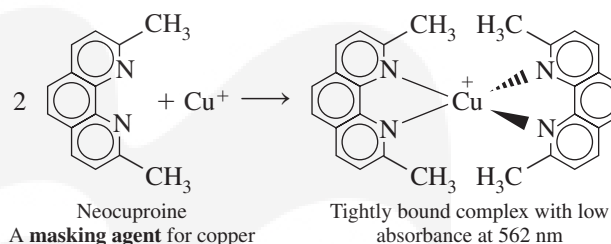
**FIGURE 18-9** Calibration curve showing the validity of Beer's law for the  $\text{Fe(II)(ferrozine)}_3^{4-}$  complex used in serum iron determination. Each sample was diluted to a final volume of 5.00 mL. Therefore, 1.00  $\mu\text{g}$  of iron is equivalent to a concentration of  $3.58 \times 10^{-6} \text{ M}$ .

is due to the color of uncomplexed ferrozine plus the color caused by the iron impurities in the reagents and glassware. The absorbance of the blank should be small compared to that of the sample and standards; otherwise, it will contribute to the overall uncertainty and degrade the detection limit. *Subtract the blank absorbance from the absorbance of samples and standards before doing any calculations.*

Prepare a series of iron standards for a *calibration curve* (Figure 18-9) to show that Beer's law is obeyed. Standards are prepared by the same procedure as unknowns. The absorbance of the unknown should fall within the region covered by the standards. If the absorbance of the unknown is too high, dilute the unknown to bring it within range. Pure iron wire with a shiny, rust-free surface is dissolved in acid to prepare standards (Appendix K). Ferrous ammonium sulfate ( $\text{Fe(NH}_4)_2(\text{SO}_4)_2 \cdot 6\text{H}_2\text{O}$ ) and ferrous ethylenediammonium sulfate ( $\text{Fe(H}_2\text{NCH}_2\text{CH}_2\text{NH}_3)_2(\text{SO}_4)_2 \cdot 4\text{H}_2\text{O}$ ) are suitable standards for less accurate work.

If unknowns and standards are prepared with identical volumes, then the quantity of iron in the unknown can be calculated from the least-squares equation for the calibration line. For example, in Figure 18-9, if the unknown has an absorbance of 0.357 (after subtracting the absorbance of the blank), the sample contains 3.59  $\mu\text{g}$  of iron.

Validation tests of the procedure with a certified reference material for iron in human serum revealed that results were about 10% high. This bias was caused by serum copper that also forms a colored complex with ferrozine. Interference is eliminated if neocuproine or thiourea is added during the complexation step. These reagents **mask**  $\text{Cu}^+$  by forming strong complexes that prevent  $\text{Cu}^+$  from reacting with ferrozine.



### EXAMPLE Serum Iron Analysis

Serum iron and standard iron solutions were analyzed as follows:

- Step 0** Turn on spectrophotometer, and allow it to warm up to stabilize the light source.
- Step 1** To 1.00 mL of serum, add 2.00 mL of reducing agent and 2.00 mL of acid to reduce and release Fe from transferrin.
- Step 2** Precipitate proteins with 1.00 mL of 30 wt% trichloroacetic acid. Centrifuge the mixture to remove protein.
- Step 3** Transfer 4.00 mL of supernatant liquid to a fresh test tube and add 1.00 mL of solution containing 2  $\mu\text{mol}$  of ferrozine and neocuproine plus 3 mmol of sodium acetate. The acetate reacts with acid to form a buffer with  $\text{pH} \approx 5$ . Measure the absorbance after 10 min.
- Step 4** To prepare the blank, use 1.00 mL of deionized water in place of serum.
- Step 5** To establish each point on the calibration curve in Figure 18-9, use 1.00 mL of standard containing 2–9  $\mu\text{g}$  Fe in place of serum.

The blank absorbance was 0.038 at 562 nm in a 1.000-cm cell. A serum sample had an absorbance of 0.129. After the blank was subtracted from each standard absorbance, the points in Figure 18-9 were obtained. The least-squares line through the standard points is

$$\text{Corrected absorbance} = 0.067_0 \times (\mu\text{g Fe in initial sample}) + 0.001_5$$

According to Beer's law, the intercept should be 0, not 0.001<sub>5</sub>. We will use the small, observed intercept for our analysis. Find the concentration of iron in the serum.

**Solution** Rearranging the least-squares equation of the calibration line and inserting the corrected absorbance (observed – blank = 0.129 – 0.038 = 0.091) of unknown, we find

$$\mu\text{g Fe in unknown} = \frac{\text{absorbance} - 0.001_5}{0.067_0} = \frac{0.091 - 0.001_5}{0.067_0} = 1.33_6 \mu\text{g}$$

To find the uncertainty in  $\mu\text{g Fe}$ , use Equation 4-27.

The concentration of Fe in the serum is

$$[\text{Fe}] = \text{moles of Fe/liters of serum} \\ = \left( \frac{1.33_6 \times 10^{-6} \text{ g Fe}}{55.845 \text{ g Fe/mol Fe}} \right) / (1.00 \times 10^{-3} \text{ L}) = 2.39 \times 10^{-5} \text{ M}$$

**TEST YOURSELF** If the observed absorbance is 0.200 and the blank absorbance is 0.049, what is the concentration of Fe ( $\mu\text{g/mL}$ ) in the serum? (**Answer:**  $2.23 \mu\text{g/mL}$ )

### EXAMPLE Preparing Standard Solutions

Points for the calibration curve in Figure 18-9 were obtained by using 1.00 mL of standard containing ~2, 3, 4, 6, 8, and 9  $\mu\text{g}$  Fe in place of serum. A stock solution was made by dissolving 1.086 g of pure, clean (Appendix K) iron wire in 40 mL of 12 M HCl and diluting to 1.000 L with  $\text{H}_2\text{O}$ . This solution contains 1.086 g Fe/L in 0.48 M HCl. How can we make Fe standards in ~0.1 M HCl for the calibration curve from this stock solution? (HCl prevents precipitation of  $\text{Fe}(\text{OH})_3$ .)

**Solution** Stock solution contains  $1.086 \text{ g Fe/L} = 1.086 \text{ mg Fe/mL} = 1\,086 \mu\text{g Fe/mL}$ . We can make a ~2  $\mu\text{g Fe/mL}$  standard by a 2:1000 dilution to give  $\frac{2 \text{ mL}}{1\,000 \text{ mL}} (1\,086 \mu\text{g Fe/mL}) = 2.172 \mu\text{g Fe/mL}$ . Transfer 2.00 mL of stock solution with a Class A transfer pipet into a 1-L volumetric flask and dilute to volume with 0.1 M HCl. In a similar manner use Class A transfer pipets to dilute the following volumes to 1 L with 0.1 M HCl:

Volume of stock solution (mL)	Class A pipet	Fe delivered ( $\mu\text{g}$ )
3.00	3 mL	$3 \times 1.086 = 3.25_8$
4.00	4 mL	$4.34_4$
6.00	$2 \times 3 \text{ mL}$	$6.51_6$
8.00	$2 \times 4 \text{ mL}$	$8.68_8$
9.00	(5 mL + 4 mL) or ( $3 \times 3 \text{ mL}$ )	$9.77_4$

Glassware (including pipets) for this procedure must be acid washed (page 35) to remove traces of metal from glass surfaces.

**TEST YOURSELF** Dilution by mass is more accurate than dilution by volume. A stock solution contains 1.044 g Fe/kg solution in 0.48 M HCl. How many  $\mu\text{g Fe/g}$  solution are in a solution made by mixing 2.145 g stock solution with 243.27 g 0.1 M HCl? (**Answer:**  $9.125 \mu\text{g Fe/g}$ )

$$\left( \frac{1.086 \text{ g Fe}}{\text{L}} \right) \left( \frac{1\,000 \text{ mg/g}}{1\,000 \text{ mL/L}} \right) = \left( \frac{1.086 \text{ mg Fe}}{\text{mL}} \right) \\ \left( \frac{1.086 \text{ mg Fe}}{\text{mL}} \right) \left( \frac{1\,000 \mu\text{g Fe}}{\text{mg Fe}} \right) = \left( \frac{1\,086 \mu\text{g Fe}}{\text{mL}} \right)$$

*Serial dilution*—a sequence of consecutive dilutions—is an important means to reduce the generation of large volumes of chemical waste by using smaller volumes for dilution.

**Serial dilution** is a sequence of consecutive dilutions.

### EXAMPLE Serial Dilution with Limited Glassware

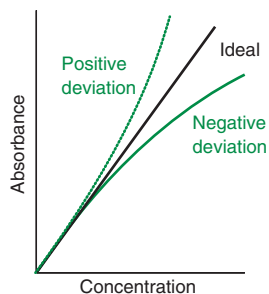
Starting from a stock solution containing 1 000  $\mu\text{g Fe/mL}$  in 0.5 M HCl, how could you make a 3  $\mu\text{g Fe/mL}$  standard in 0.1 M HCl if you have only 100-mL volumetric flasks and 1-, 5-, and 10-mL transfer pipets?

**Solution** One way to solve this problem is by these two steps:

1. Pipet three 10.00-mL portions of stock solution into a 100-mL flask and dilute to the mark with 0.1 M HCl.  $[\text{Fe}] = \left( \frac{30 \text{ mL}}{100 \text{ mL}} \right) (1\,000 \mu\text{g Fe/mL}) = 300 \mu\text{g Fe/mL}$ .
2. Transfer 1.00 mL of solution from step 1 to a 100-mL flask and dilute to the mark with 0.1 M HCl.  $[\text{Fe}] = \left( \frac{1 \text{ mL}}{100 \text{ mL}} \right) (300 \mu\text{g Fe/mL}) = 3 \mu\text{g Fe/mL}$ .

**TEST YOURSELF** Describe a different set of dilutions to produce 3  $\mu\text{g Fe/mL}$  from 1 000  $\mu\text{g Fe/mL}$ . (**Answer:** Dilute 10 mL of stock to 100 mL to get  $[\text{Fe}] = 100 \mu\text{g Fe/mL}$ . Dilute  $3 \times 1.00 \text{ mL}$  of the new solution up to 100 mL to get 3  $\mu\text{g Fe/mL}$ .)

Beer's law works for monochromatic radiation passing through a dilute solution in which the absorbing species is not participating in a concentration-dependent equilibrium.



Causes of deviations from Beer's law:

**Positive deviation:** chemical effects

**Negative deviation:** chemical effects  
polychromatic light  
stray light

Instrumental deviations from Beer's law are discussed in greater detail in Section 20-2.

Serial dilution propagates any systematic error in the stock solution. Quality control samples with known analyte content can detect systematic error.

## When Beer's Law Fails

Beer's law states that absorbance is proportional to the concentration of the absorbing species. It applies to *monochromatic* radiation, and it works very well for dilute solutions ( $\leq 0.01$  M) of most substances.

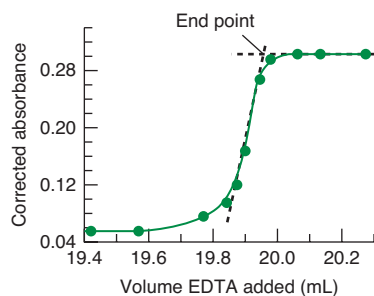
In concentrated solutions, solute molecules influence one another as a result of their proximity. At very high concentration, the solute *becomes* the solvent. Properties of a molecule are not exactly the same in different environments. Nonabsorbing solutes in a solution can also interact with the absorbing species and alter the absorptivity.

If the absorbing molecule participates in a concentration-dependent chemical equilibrium, the absorptivity changes with concentration. For example, in concentrated solution, a weak acid, HA, may be mostly undissociated. As the solution is diluted, dissociation increases. If the absorptivity of  $A^-$  is not the same as that of HA, the solution will appear not to obey Beer's law as it is diluted. Buffering samples and standards minimizes this error. Confirm that the spectral profile does not change with analyte concentration. Chemical nonidealities may cause either a positive or negative deviation from Beer's law.

Beer's law applies to monochromatic light, but spectrophotometers provide a range of wavelengths centered around the selected wavelength. Beer's law is obeyed when absorbance is constant across the selected waveband, such as if we measure Fe at  $562 \pm 5$  nm in Figure 18-8. If, instead, we measure Fe at  $600 \pm 5$  nm, molar absorptivity changes significantly across the bandwidth. Apparent absorbance depends on the integrated transmittance over the bandwidth. Wavelengths within the bandwidth with lower absorbance transmit disproportionately more light, due to the logarithmic relation between absorbance and transmittance. This results in lower apparent absorbances. Also, the negative deviation from Beer's law due to polychromatic light is greater at higher absorbance where little light is transmitted, causing curvature in the calibration plot. In practice, "monochromatic" means that the bandwidth of the light must be substantially smaller than the width of the absorption band of the chromophore.<sup>16</sup>

Beer's law assumes all light reaching the detector passed through the sample. If stray light from the room or from within the spectrophotometer reaches the detector, a negative deviation from Beer's law results, so cuvette compartments must be tightly closed. Cuvettes should contain sufficient liquid that none of the light beam passes *over* the solution. With micro cells, a misaligned light beam might pass through the glass walls on either side of the solution chamber. Blackened cells such as the flow cell in Figure 18-5 minimize this source of stray light. Fluorescence from the sample also acts as stray light. The effect of stray light is reduced by working at lower absorbances.

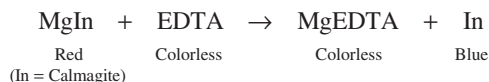
You should plot calibration data, and inspect the *residuals*—the vertical deviation between the measured value and the y value predicted by the least-squares line (page 106). A pattern such as curvature in the residuals suggests that Beer's law is failing, and the procedure should be refined. Chemical deviations can be reduced by diluting or buffering the standards and samples. Instrumental deviations are reduced by working at lower absorbance or by adjusting instrumental settings (page 521). If the calibration still exhibits curvature, either a smaller concentration range or a quadratic least-squares fit may be more appropriate (Appendix C).



**FIGURE 18-10** Spectrophotometric titration of calcium and magnesium with EDTA at pH 10. Absorbance of free Calmagite indicator (In) is monitored at 610 nm. The initial absorbance of the solution is due to slight absorbance of MgIn at 610 nm. See spectra in Demonstration 12-1. [Data from G. Kiema, University of Alberta.]

## 18-5 Spectrophotometric Titrations

In a **spectrophotometric titration**, we monitor changes in absorbance during a titration to tell when the equivalence point has been reached. To determine water hardness (Box 12-2) the calcium and magnesium in water are titrated with ethylenediaminetetraacetic acid (EDTA) at pH 10 with Calmagite indicator (Table 12-3). The end point reaction is



As the equivalence point is approached, the color changes from the red of the MgIn complex, to purple—a mixture of red and blue—to the blue of free In (Color Plate 9). The equivalence point is when the color is pure blue, with no hint of purple. Detecting this end point is one of the largest sources of error in this titration.

Figure 18-10 shows the spectrophotometric titration of a 10.00-mL sample with 0.015 23 M EDTA buffered to pH 10. Absorbance is monitored directly in the beaker with an



optical immersion probe (Figure 18-11). A survey titration using visual detection determined the end point was near 20 mL. For the spectrophotometric titration, titrant was added to within 1 mL of the end point. Near the end point EDTA displaces  $\text{Mg}^{2+}$  from  $\text{MgIn}$ . The freed In causes absorbance to increase at 610 nm. When all metal is bound by EDTA, all indicator is in the free form and the curve levels off. The extrapolated intersection of the two straight portions of the titration curve at  $19.95_8$  mL is the end point. Titrant should be added dropwise near the end point, and a number of readings taken after the end point, to enable accurate extrapolation. The quantity of EDTA required for complete reaction is  $(0.199_5 \text{ L}) \times (0.01523 \text{ mol/L}) = 3.040 \text{ mmol}$ . Precision for the spectrophotometric titration is 0.4–0.5%.

To construct the graph in Figure 18-10, dilution must be considered because the volume is different at each point. Each point on the graph represents the absorbance that would be observed *if the solution had not been diluted from its original volume of 10.00 mL*.

$$\text{Corrected absorbance} = \left( \frac{\text{total volume}}{\text{initial volume}} \right) (\text{observed absorbance}) \quad (18-7)$$

### EXAMPLE Correcting Absorbance for the Effect of Dilution

The absorbance measured after adding 19.90 mL of EDTA to 10.00 mL of drinking water was 0.057. Calculate the corrected absorbance that should be plotted in Figure 18-10.

**Solution** The total volume was  $10.00 + 19.90 = 29.90$  mL. If the volume had remained 10.00 mL, the absorbance would have been greater than 0.057 by a factor of  $29.90/10.00$ .

$$\text{Corrected absorbance} = \left( \frac{29.90 \text{ mL}}{10.00 \text{ mL}} \right) (0.057) = 0.170$$

The corrected absorbance plotted at 19.90 mL in Figure 18-10 is 0.170.

**TEST YOURSELF** In a different titration, the absorbance after adding 8.94 mL of EDTA to 5.00 mL of drinking water was 0.105. Find the corrected absorbance. (**Answer:** 0.293)

Small volumes of sample can be titrated directly in a stirred cuvette within a spectrophotometer. Addition of titrant with a Hamilton syringe (Figure 2-15) provides 1–2% precision.

## 18-6 What Happens When a Molecule Absorbs Light?

When a molecule absorbs a photon, the molecule is promoted to a more energetic *excited state* (Figure 18-3). Conversely, when a molecule emits a photon, the energy of the molecule falls by an amount equal to the energy of the emitted photon.

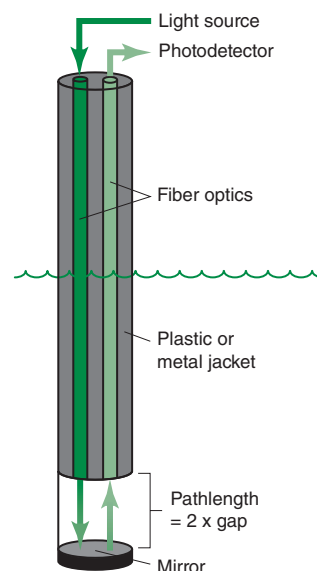
For example, consider formaldehyde in Figure 18-12. In its ground state, the molecule is planar, with a double bond between carbon and oxygen. From the electron dot description of formaldehyde, we expect two pairs of nonbonding electrons to be localized on the oxygen atom. The double bond consists of a sigma bond between carbon and oxygen and a pi bond made from the  $2p_y$  (out-of-plane) atomic orbitals of carbon and oxygen.

### Electronic States of Formaldehyde

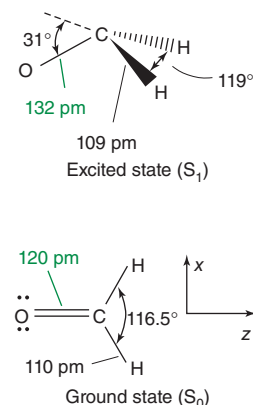
**Molecular orbitals** describe the distribution of electrons in a molecule, just as *atomic orbitals* describe the distribution of electrons in an atom. In the molecular orbital diagram for formaldehyde in Figure 18-13, one of the nonbonding orbitals of oxygen is mixed with the three sigma bonding orbitals. These four orbitals, labeled  $\sigma_1$  through  $\sigma_4$ , are each occupied by a pair of electrons with opposite spin (spin quantum numbers =  $+\frac{1}{2}$  and  $-\frac{1}{2}$ ). At higher energy is an occupied pi bonding orbital ( $\pi$ ), made of the  $p_y$  atomic orbitals of carbon and oxygen. The highest energy occupied orbital is the nonbonding orbital ( $n$ ), composed principally of the oxygen  $2p_x$  atomic orbital. The lowest energy unoccupied orbital is the pi antibonding orbital ( $\pi^*$ ). When occupied by electrons, this antibonding orbital causes repulsion between the carbon and oxygen atoms.

In an **electronic transition**, an electron from one molecular orbital transfers to another orbital, with a concomitant increase or decrease in the energy of the molecule. The lowest energy electronic transition of formaldehyde promotes a nonbonding ( $n$ ) electron to the antibonding pi orbital ( $\pi^*$ ).<sup>17</sup> There are two possible transitions, depending on the spin

Precision of spectrophotometric analyses:  
Calibration curve 1–3%  
Titration 0.3–1%

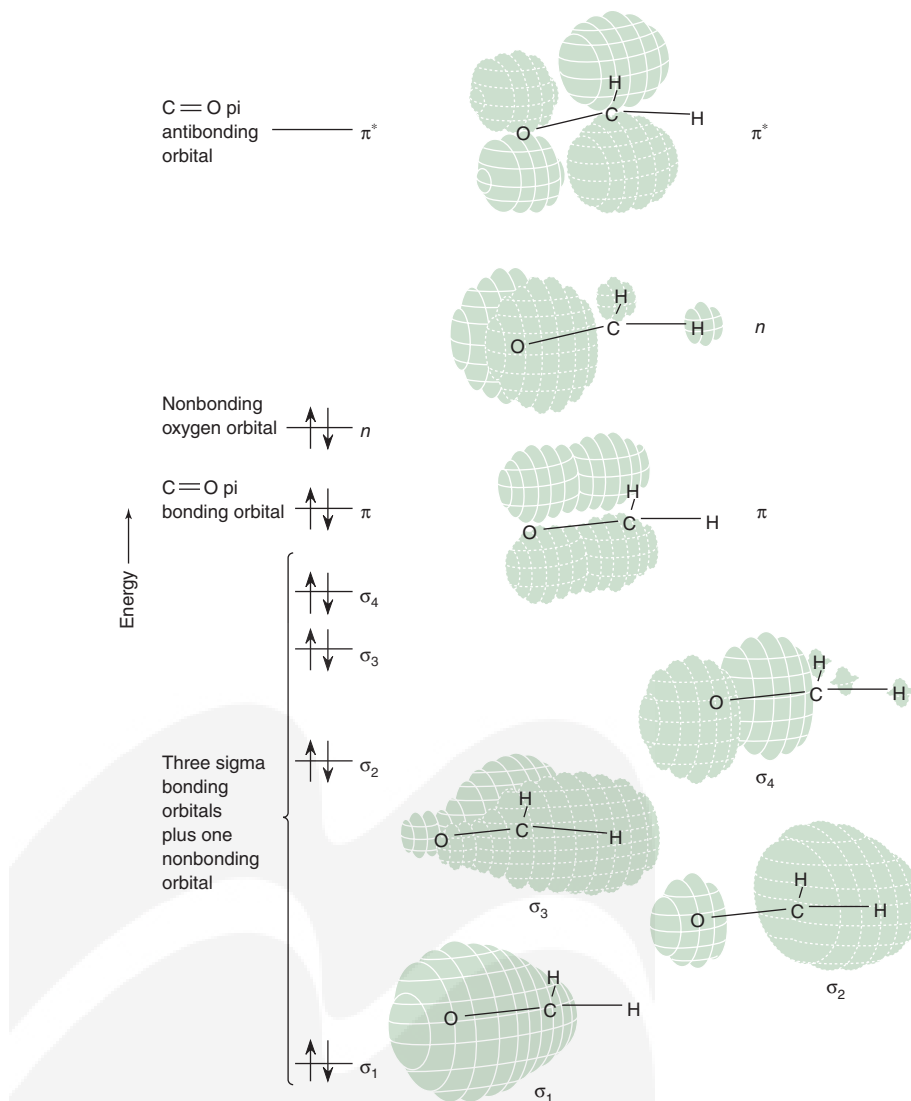


**FIGURE 18-11** Optical fiber probe, also called an *optode*, is immersed in sample solution. Light from spectrophotometer is guided by an optical fiber (Section 20-4) to pass through sample solution to a mirror and back. Transmitted light is collected by a second optical fiber and directed to a photodetector.

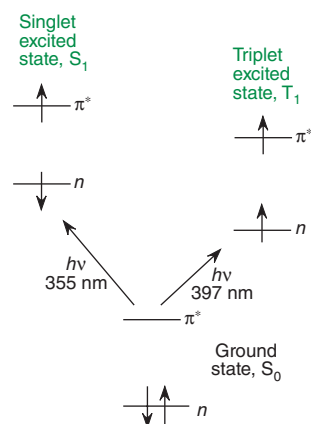


**FIGURE 18-12** Geometry of formaldehyde in its ground state  $S_0$  and lowest excited singlet state  $S_1$ .





**FIGURE 18-13** Molecular orbital diagram of formaldehyde, showing energy levels and orbital shapes. The coordinate system of the molecule was shown in Figure 18-12. [Information from W. L. Jorgensen and L. Salem, *The Organic Chemist's Book of Orbitals* (New York: Academic Press, 1973).]



**FIGURE 18-14** Two possible electronic states arise from an  $n \rightarrow \pi^*$  transition. The terms “singlet” and “triplet” are used because a triplet state splits into three slightly different energy levels in a magnetic field, but a singlet state is not split.

quantum numbers in the excited state (Figure 18-14). The state in which the spins are opposed is called a **singlet state**. If the spins are parallel, we have a **triplet state**.

The lowest energy excited singlet and triplet states are called  $S_1$  and  $T_1$ , respectively. In general,  $T_1$  has lower energy than  $S_1$ . In formaldehyde, the transition  $n \rightarrow \pi^*(T_1)$  requires absorption of visible light with a wavelength of 397 nm. The  $n \rightarrow \pi^*(S_1)$  transition occurs when ultraviolet radiation with a wavelength of 355 nm is absorbed.

With an electronic transition near 397 nm, you might expect from Table 18-1 that formaldehyde appears green-yellow. In fact, formaldehyde is colorless because the probability of undergoing a transition between singlet and triplet states, such as  $n(S_0) \rightarrow \pi^*(T_1)$ , is exceedingly small. Formaldehyde absorbs so little light at 397 nm that our eyes do not detect any absorbance at all. Singlet-to-singlet transitions such as  $n(S_0) \rightarrow \pi^*(S_1)$  are much more probable, so the ultraviolet absorption is more intense.

Although formaldehyde is planar in its ground state, it has a pyramidal structure in both the  $S_1$  (Figure 18-12) and  $T_1$  excited states. Promotion of a nonbonding electron to an antibonding C—O orbital lengthens the C—O bond and changes the molecular geometry.

### Vibrational and Rotational States of Formaldehyde

Absorption of visible or ultraviolet radiation promotes electrons to higher energy orbitals in formaldehyde. Infrared and microwave radiation are not energetic enough to induce electronic transitions, but they can change the vibrational or rotational motion of the molecule.

Each of the four atoms of formaldehyde can move along three axes in space, so the entire molecule can move in  $4 \times 3 = 12$  different ways. Three of these motions correspond to translation of the entire molecule in the  $x$ ,  $y$ , and  $z$  directions. Another three motions correspond to rotation about  $x$ -,  $y$ -, and  $z$ -axes placed at the center of mass of the molecule. The remaining six motions are vibrations shown in Figure 18-15.

When formaldehyde absorbs an infrared photon with a wavenumber of  $1251\text{ cm}^{-1}$  ( $= 14.97\text{ kJ/mol}$ ), the asymmetric bending vibration in Figure 18-15 is stimulated: Oscillations of the atoms increase in amplitude, and the energy of the molecule increases.

Spacings between rotational energy levels of a molecule are smaller than vibrational energy spacings. A molecule in the rotational ground state could absorb microwave photons with energies of  $0.029\text{ kJ/mol}$  or  $0.087\text{ kJ/mol}$  (wavelengths of  $4.115$  or  $1.372\text{ mm}$ ) to be promoted to the two lowest excited states. Absorption of microwave radiation causes the molecule to rotate faster than it does in its ground state.

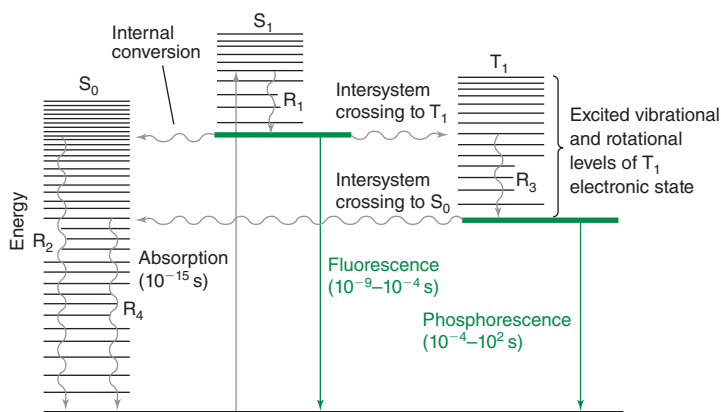
### Combined Electronic, Vibrational, and Rotational Transitions

In general, when a molecule absorbs light having sufficient energy to cause an electronic transition, **vibrational** and **rotational transitions**—that is, changes in the vibrational and rotational states—occur as well. Formaldehyde can absorb one photon with just the right energy to cause the following simultaneous changes: (1) a transition from the  $S_0$  to the  $S_1$  electronic state, (2) a change in vibrational energy from the ground vibrational state of  $S_0$  to an excited vibrational state of  $S_1$ , and (3) a transition from one rotational state of  $S_0$  to a different rotational state of  $S_1$ . These energy changes are evident in the spectrum of formaldehyde in the gas phase in the opener of this chapter. The absorption at  $355\text{ nm}$  (highlighted with \*) is the transition from the  $S_0$  to the  $S_1$  electronic state with the smallest change in vibrational energy. Higher energy (lower wavelength) absorption bands correspond to  $S_0$  to  $S_1$  transitions with simultaneous changes in vibrational energy. The width of each vibrational band is due to unresolved rotational transitions accompanying the  $S_0$  to  $S_1$  electronic and vibrational transitions.

Electronic absorption bands in solution are usually broad (Figure 18-8). Interactions with solvent molecules perturb the vibrational and rotational energy levels of individual analyte molecules. These interactions blur the rotational and even vibrational features of the spectrum, particularly in polar solvents such as water, resulting in broad featureless absorption bands. A molecule can absorb photons with a wide range of energies and still be promoted from the ground electronic state to one particular excited electronic state.

### What Happens to Absorbed Energy?

Suppose that absorption promotes a molecule from the ground electronic state,  $S_0$ , to a vibrationally and rotationally excited level of the excited electronic state  $S_1$  (Figure 18-16). Usually, the first process after absorption is *vibrational relaxation* to the lowest vibrational level of  $S_1$ . In this *nonradiative* (no light emitted) transition, labeled  $R_1$  in Figure 18-16, vibrational energy is transferred to other molecules (solvent, for example) through collisions.

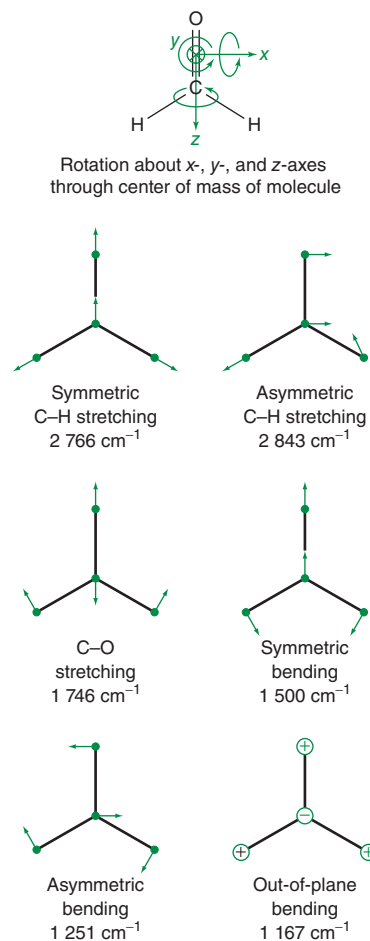


**FIGURE 18-16** Jablonski diagram showing physical processes that can occur after a molecule absorbs an ultraviolet or visible photon.  $S_0$  is the ground electronic state.  $S_1$  and  $T_1$  are the lowest excited singlet and triplet electronic states. Straight arrows represent processes involving photons, and wavy arrows are nonradiative transitions.  $R$  denotes vibrational relaxation. Absorption could terminate in any of the vibrational levels of  $S_1$ , not just the one shown. Fluorescence and phosphorescence can terminate in any of the vibrational levels of  $S_0$ .

A nonlinear molecule with  $n$  atoms has  $3n - 6$  vibrational modes and three rotations. A linear molecule can rotate about only two axes; it therefore has  $3n - 5$  vibrational modes and two rotations.

Your microwave oven heats food by transferring rotational energy to water molecules in the food.

Vibrational transitions usually involve simultaneous rotational transitions.



**FIGURE 18-15** The six modes of vibration of formaldehyde. The wavenumber of infrared radiation needed to stimulate each kind of motion is given in units of reciprocal centimeters,  $\text{cm}^{-1}$ . The molecule rotates about  $x$ -,  $y$ -, and  $z$ -axes located at the center of mass near the middle of the  $\text{C}=\text{O}$  bond.

**Internal conversion** is a nonradiative transition between states with the same spin (e.g.,  $S_1 \rightarrow S_0$ ).

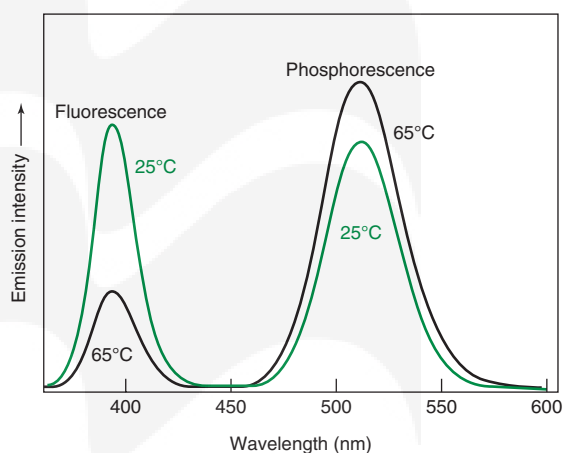
**Intersystem crossing** is a nonradiative transition between states with different spin (e.g.,  $T_1 \rightarrow S_0$ ).

**Fluorescence:** emission of a photon during a transition between states with the same spin (e.g.,  $S_1 \rightarrow S_0$ ).

**Phosphorescence:** emission of a photon during a transition between states with different spin (e.g.,  $T_1 \rightarrow S_0$ ).

**FIGURE 18-17** Emission spectra showing that phosphorescence comes at lower energy (longer wavelength) than fluorescence from the same molecule. Intersystem crossing from  $S_1$  to  $T_1$  is slow. Increasing temperature in this case results in faster intersystem crossing and greater phosphorescence.

[Data from A. C. Durrell, G. E. Keller, Y.-C. Lam, J. Sýkora, A. Vlček, Jr., and H. B. Gray, "Structural Control of  $^1A_{2u}$ -to- $^3A_{2u}$  Intersystem Crossing in Diplatinum(II,II) Complexes," *J. Am. Chem. Soc.* **2012**, 134, 14201.]

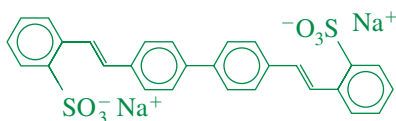


Compounds which can fluoresce or phosphoresce are relatively rare. Molecules that are loose and floppy with many vibrational and rotational modes generally relax from the excited state by nonradiative transitions. In more rigid molecules, nonradiative pathways may not be available, and the excited molecule can only relax by spontaneous emission of a photon—but this takes time. The *lifetime* of fluorescence is  $10^{-9}$  to  $10^{-4}$  s. The lifetime of phosphorescence is longer ( $10^{-4}$  to  $10^2$  s) because phosphorescence involves a change in spin quantum numbers (from two unpaired electrons to no unpaired electrons), which is improbable. A few materials, such as strontium aluminate doped with europium and dysprosium ( $\text{SrAl}_2\text{O}_4\text{:Eu:Dy}$ ), phosphoresce for *hours* after exposure to light.<sup>18</sup> One application for this material is in signs leading to emergency exits when power is lost.

## 18-7 Luminescence

Fluorescence and phosphorescence are examples of **luminescence**, which is emission of light from an excited state of a molecule. Luminescence is inherently more sensitive than absorption. Imagine yourself in a stadium at night; the lights are off, but each of the 50 000 raving fans is holding a lighted cell phone. If 500 people turn off their screens, you will hardly notice the difference. Now imagine that the stadium is completely dark; then 500 people suddenly turn on their candle app. In this case, the effect is dramatic. The first example is analogous to changing

Most white fabrics fluoresce. Just for fun, turn on an ultraviolet lamp in a darkened room containing several people (*but do not look directly at the lamp*). You will discover emission from white fabrics (shirts, pants, shoelaces, unmentionables) containing fluorescent compounds to enhance whiteness. You might see fluorescence from teeth and from recently bruised areas of skin that show no surface damage. Tonic water, turmeric spice, and highlighter ink are among other everyday items that fluoresce. Glow-in-the-dark toys exhibit phosphorescence. Many fun demonstrations and videos of fluorescence and phosphorescence have been developed.<sup>19</sup>



A fluorescent whitener from laundry detergent

But fluorescence is not just about pretty colors. In the year 2000, about 20% of global energy consumption was for lighting. After a century of development, incandescent light bulbs had only improved 10-fold in efficiency. Fluorescent bulbs were more efficient but contained toxic Hg. Light-emitting diodes held the promise of much greater efficiency and longer lifetimes but were not available across the full visible spectrum. White light needs a balance of blue, green, and red light to appear natural.

After considerable research—ultimately leading to the 2014 Nobel Prize in Physics—white light-emitting diodes surpassed the efficiency of fluorescent lights.<sup>20</sup> White light-emitting diodes must balance absorbance, transmittance, and phosphorescence. Blue light is generated by an emitting diode, as in panel *a*, made of semiconductors such as gallium nitride and indium gallium nitride. Some

of the blue light is absorbed by the  $\text{Ce}^{3+}$ -activated yttrium aluminum garnet ( $\text{Y}_3\text{Al}_5\text{O}_{12}:\text{Ce}^{3+}$ ) phosphor within the diode in panel *b*. Phosphorescence produces a broad yellow emission, which combined with the transmitted blue light, provides a near-white light, shown in panel *c*. But the lack of red emission means illuminated objects did not appear their natural color. A red apple that appears black would not look tasty. Truer white light was achieved by adding traces of gadolinium to give the phosphor a stronger red component, while gallium doping made the emission more green. Widespread use of white light-emitting diodes can cut our electricity use for lighting in half—preventing 200 million metric tons of  $\text{CO}_2$  emission per year. Development of new phosphor-diode combinations that provide a more natural white light with greater energy efficiency is an active area of research.

#### Lamp

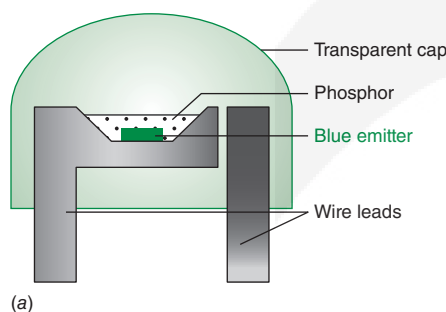
Approximate efficiency  
(lumens per watt)<sup>a, b</sup>

Edison incandescent bulb with carbon filament	1–2
Incandescent light bulb with tungsten filament	10–18
Compact fluorescent lamp	50–70
Long fluorescent tube	65–95
White light-emitting diode (LED) <sup>c</sup>	>200

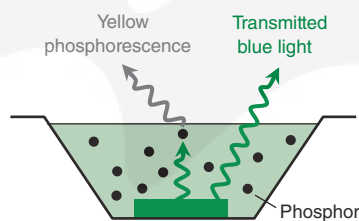
*a.* J. Cho, J. H. Park, J. K. Kim, and E. F. Schubert, “White Light-Emitting Diodes,” *Laser Photonics Rev.* **2017**, 1600147.

*b.* Lumen (lm) is a measure of luminous flux. 1 lm = radiant energy emitted in a solid angle of 1 steradian (sr) from a source that radiates 1/683 W/sr uniformly in all directions at a frequency of 540 THz (near the middle of the visible spectrum).

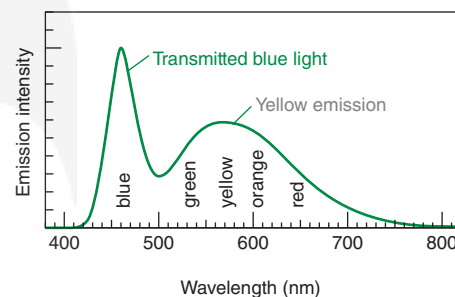
*c.* LED lamp lifetime  $\approx 10^5$  h; fluorescent lamp lifetime  $\approx 10^4$  h; incandescent lamp lifetime  $\approx 10^3$  h



(a)



(b)



(c)

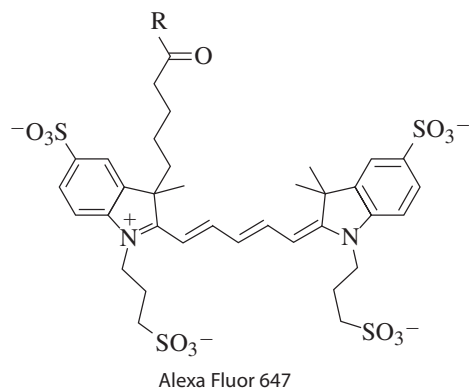
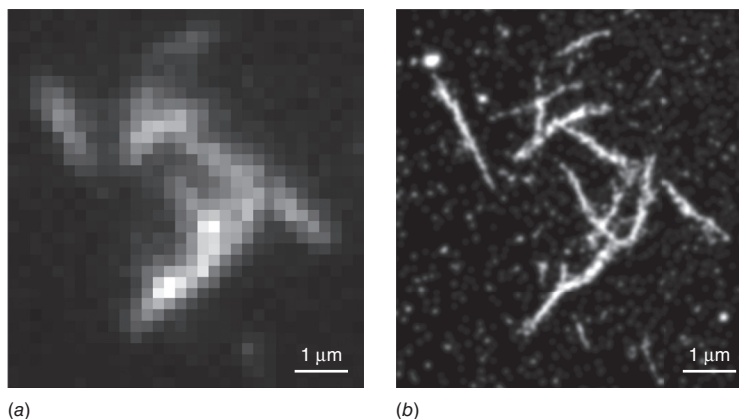
(a) Structure of white light-emitting diode (b) Blue light is from the diode and yellow is phosphorescence when blue light excites the phosphor. (c) Emission spectrum of early white light-emitting diode showing blue at 460 nm generated by the diode and a broad phosphorescence emission from green to red from phosphor. [Information from J. Cho, J. H. Park, J. K. Kim, and E. F. Schubert, “White Light-Emitting Diodes,” *Laser Photonics Rev.* **2017**, 1600147.]

transmittance from 100 to 99%, which is equivalent to an absorbance of  $-\log 0.99 = 0.0044$ . It is hard to measure such a small absorbance because the background is so bright. The second example is analogous to observing fluorescence from 1% of the molecules in a sample. Against a black background, fluorescence is striking.

Luminescence is sensitive enough to observe *single molecules*.<sup>21</sup> Each single fluorescent molecule can act as a light source about 1 nm in size. This enabled microscopy at dimensions below the optical diffraction limit, an achievement recognized by the 2014 Nobel Prize in Chemistry.<sup>22</sup> Diffraction caused by the wave properties of light means that traditional optical microscopy cannot resolve features separated by less than about half the wavelength of light. Figure 18-18 shows a diffraction-limited microscope image and a super-resolution fluorescence image of the protein huntingtin with the highly fluorescent label Alexa



**FIGURE 18-18** Aggregates of huntingtin, a protein implicated in Huntington's disease, observed by (a) diffraction-limited microscopy and (b) super-resolution fluorescence microscopy. Labeling huntingtin with highly fluorescent Alexa Fluor 647 makes microscopy possible. [Photo courtesy of Whitney C. Duim, University of California, Davis, data from W. C. Duim, Y. Jiang, K. Shen, J. Frydman, and W. E. Moerner, "Super-Resolution Fluorescence of Huntingtin Reveals Growth of Globular Species into Short Fibers and Coexistence of Distinct Aggregates." *ACS Chem. Biol.* **2014**, 9, 2767–2778.]



Electronic transitions usually involve simultaneous vibrational and rotational transitions.

Fluor 647. Mutations in huntingtin gene result in misfolded protein whose aggregation causes Huntington's disease. Single-molecule detection, made possible by the sensitivity of fluorescence, enabled characterization of the aggregation pathway from single protein molecules to fiber assemblies.

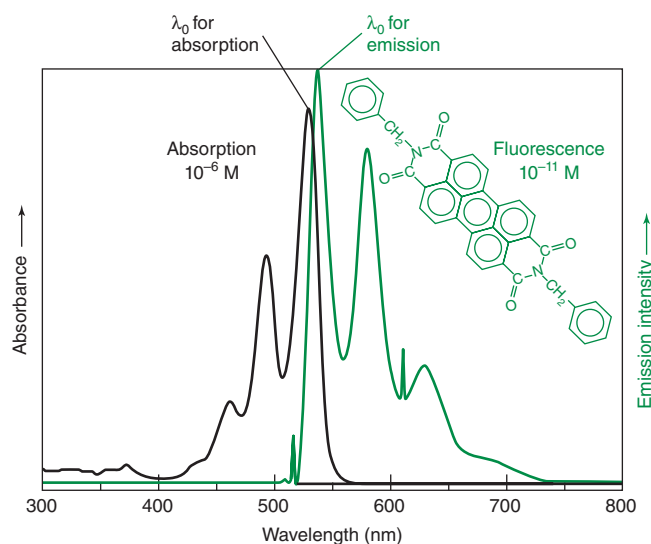
### Relation Between Absorption and Emission Spectra

Figure 18-16 shows that fluorescence and phosphorescence come at lower energy than absorption (the *excitation energy*). That is, molecules emit radiation at longer wavelengths than the radiation they absorb. Examples are shown in Figure 18-19 and Color Plates 16, 18 and 22.

Figure 18-20 explains why emission comes at lower energy than absorption and why the emission spectrum is roughly the mirror image of the absorption spectrum. In absorption, wavelength  $\lambda_0$  corresponds to a transition from the ground vibrational level of  $S_0$  to the lowest vibrational level of  $S_1$ . Absorption maxima at higher energy (shorter wavelength) correspond to the  $S_0$ -to- $S_1$  transition accompanied by absorption of one or more quanta of vibrational energy. In polar solvents, vibrational structure is usually broadened beyond recognition, and only a broad envelope of absorption is observed. In less polar or nonpolar solvents, such as dichloromethane in Figure 18-19, vibrational structure is observed.

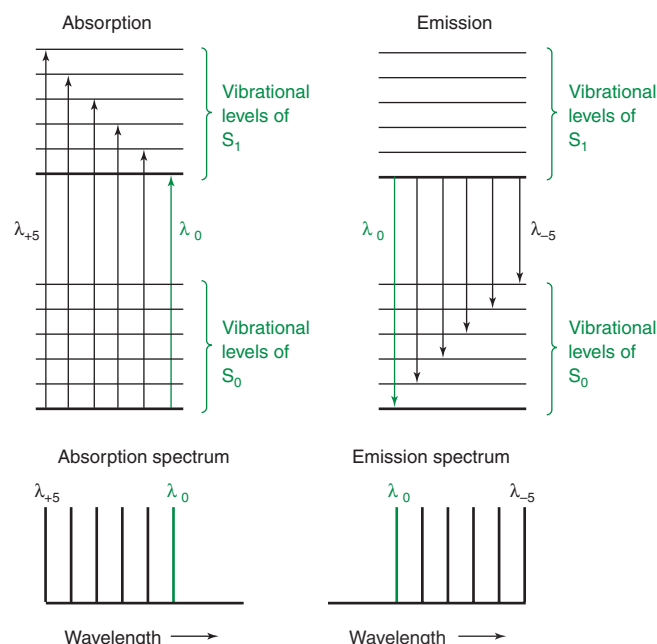
After absorption, the vibrationally excited  $S_1$  molecule relaxes to the lowest vibrational level of  $S_1$  prior to emitting any radiation. Emission from  $S_1$  in Figure 18-20 can go to any vibrational level of  $S_0$ . The highest energy transition comes at wavelength  $\lambda_0$ , with a series of peaks following at longer wavelength. Absorption and emission spectra will have an approximate mirror image relation if the spacings between vibrational levels of  $S_0$  and  $S_1$  are roughly equal and if the transition probabilities are similar.

The  $\lambda_0$  transitions in Figure 18-19 (and later in Figure 18-23) do not exactly overlap. In the emission spectrum,  $\lambda_0$  comes at slightly lower energy (longer wavelength) than in the absorption spectrum. Figure 18-21 explains the reason. A molecule absorbing radiation is initially in its electronic ground state,  $S_0$ . This molecule possesses a certain geometry and solvation.

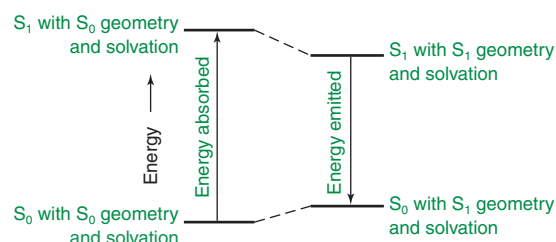


**FIGURE 18-19** Absorption (black line) and emission (colored line) of bis(benzylimido)perylene in dichloromethane solution, illustrating the approximate mirror image relation between absorption and emission. The  $10^{-11}$  M solution used for emission had an average of just 10 analyte molecules in the volume probed by the 514-nm excitation laser. [Data from P. J. G. Goulet, N. P. W. Pieczonka, and R. F. Aroca, "Overtones and Combinations in Single-Molecule Surface-Enhanced Resonance Raman Scattering Spectra," *Anal. Chem.* **2003**, 75, 1918. P. J. G. Goulet and N. P. W. Pieczonka were undergraduate researchers.]





**FIGURE 18-20** Energy-level diagram showing why vibrational structure is seen in the absorption and emission spectra and why the spectra are roughly mirror images of each other. In absorption, wavelength  $\lambda_0$  comes at lowest energy, and  $\lambda_{+5}$  is at highest energy. In emission, wavelength  $\lambda_0$  comes at highest energy, and  $\lambda_{-5}$  is at lowest energy.



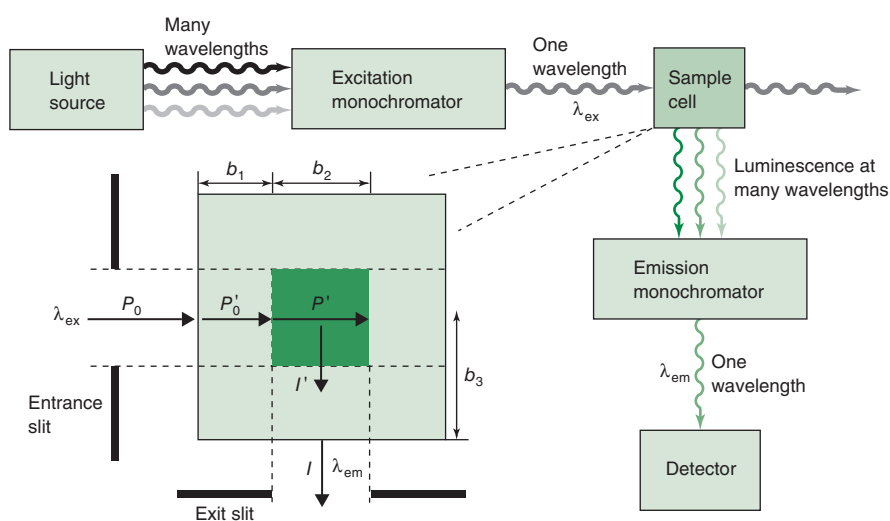
**FIGURE 18-21** Diagram showing why the  $\lambda_0$  transitions do not exactly overlap in Figures 18-19 and 18-23.

The electronic transition from  $S_0$  to excited state  $S_1$  is faster ( $\leq 10^{-15}$  s) than the vibrational motion of atoms or the translational motion of solvent molecules. In the instant after the radiation is first absorbed, the excited  $S_1$  molecule still possesses  $S_0$  geometry and solvation. Shortly after excitation, the geometry and solvation change to their most favorable values for the  $S_1$  state. This rearrangement lowers the energy of the excited molecule. When an  $S_1$  molecule fluoresces, it rapidly ( $\leq 10^{-15}$  s) returns to the  $S_0$  state with  $S_1$  geometry and solvation. This unstable configuration must have a higher energy than that of an  $S_0$  molecule with  $S_0$  geometry and solvation. The net effect in Figure 18-21 is that the  $\lambda_0$  emission energy is less than the  $\lambda_0$  excitation energy.

Electronic transitions are fast, relative to nuclear motion, so each atom has nearly the same position and momentum before and after a transition. This is called the *Franck-Condon principle*.

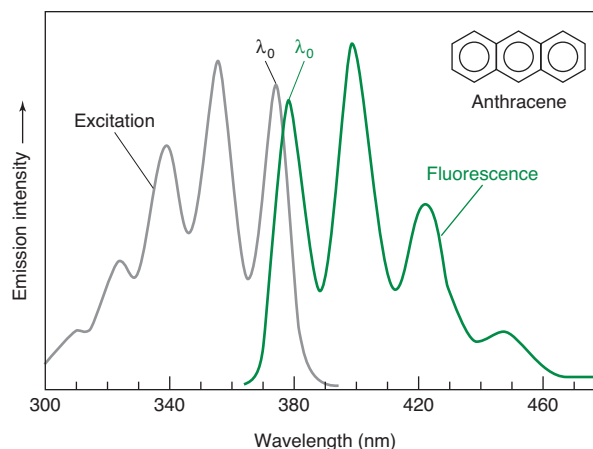
### Excitation and Emission Spectra

An emission experiment is shown in Figure 18-22. An excitation wavelength ( $\lambda_{\text{ex}}$ ) is selected by one monochromator and directed to the sample. Emission occurs at all angles relative to



**FIGURE 18-22** Essentials of a luminescence experiment. The sample is irradiated at one wavelength and emission is observed over a range of wavelengths. The excitation monochromator selects the excitation wavelength ( $\lambda_{\text{ex}}$ ) and the emission monochromator selects one wavelength at a time ( $\lambda_{\text{em}}$ ) to observe.

**FIGURE 18-23** Excitation and emission spectra of anthracene in cyclohexane have the same mirror image relationship as the absorption and emission spectra in Figure 18-19. An excitation spectrum is nearly the same as an absorption spectrum. [Data from C. M. Byron and T. C. Werner, "Experiments in Synchronous Fluorescence Spectroscopy for the Undergraduate Instrumental Chemistry Course," *J. Chem. Ed.* **1991**, 68, 433.]



the excitation beam. Luminescence is monitored through a second monochromator, usually positioned at 90° to the incident light to minimize the intensity of excitation light reaching the detector. If we hold the excitation wavelength fixed and scan through the emitted radiation, an **emission spectrum** such as the right side of Figure 18-23 is produced. An emission spectrum is a graph of emission intensity versus emission wavelength. A database with over 200 fluorescence spectra is available online.<sup>4</sup>

An **excitation spectrum** is measured by varying the excitation wavelength and measuring emitted light at one particular wavelength ( $\lambda_{em}$ ). An excitation spectrum is a graph of emission intensity versus excitation wavelength (Figure 18-23). *An excitation spectrum looks very much like an absorption spectrum* because the greater the absorbance at the excitation wavelength, the more molecules are promoted to the excited state and the more emission will be observed.

In emission spectroscopy, we measure emitted radiation, rather than the fraction of incident radiation striking the detector. Detector response and monochromator efficiency vary with wavelength, so the recorded emission spectrum is not a true profile of emitted irradiance versus emission wavelength. For quantitative analysis employing a single emission wavelength, this effect is inconsequential. Box 18-3 describes common forms of light scattering by molecules that can be confused with emission when you are interpreting an experimental spectrum.

### Luminescence Intensity

A simplified view of processes occurring during a luminescence measurement is shown in the enlarged sample cell at the lower left of Figure 18-22. We expect emission to be proportional to the irradiance absorbed by the sample. In Figure 18-22, the irradiance ( $\text{W/m}^2$ ) incident on the sample cell is  $P_0$ . Some light is absorbed over the pathlength  $b_1$ , so the irradiance striking the central region of the cell is

$$\text{Irradiance striking central region} = P'_0 = P_0 \cdot 10^{-\epsilon_{ex} b_1 c} \quad (18-8)$$

where  $\epsilon_{ex}$  is the molar absorptivity at wavelength  $\lambda_{ex}$  and  $c$  is the concentration of analyte. The irradiance of the beam when it has traveled the additional distance  $b_2$  is

$$P' = P'_0 10^{-\epsilon_{ex} b_2 c} \quad (18-9)$$

Emission intensity  $I$  is proportional to irradiance absorbed in the central region of the cell:

$$\text{Emission intensity} = I' = k' \Phi (P'_0 - P') \quad (18-10)$$

where  $k'$  is a constant of proportionality, and  $\Phi$  is the **quantum yield** which is the fraction of photons absorbed which results in luminescence. The quantum yield ranges from 0 for a nonfluorescent molecule to a maximum of 1 and depends on solution conditions (Section 19-6). Not all radiation emitted from the center of the cell in the direction of the exit slit is observed. Some is absorbed between the center and the edge of the cell. Emission intensity  $I$  emerging from the cell is given by Beer's law:

$$I = I' \cdot 10^{-\epsilon_{em} b_3 c} \quad (18-11)$$

where  $\epsilon_{em}$  is the molar absorptivity at the emission wavelength and  $b_3$  is the distance from the center to the edge of the cell.

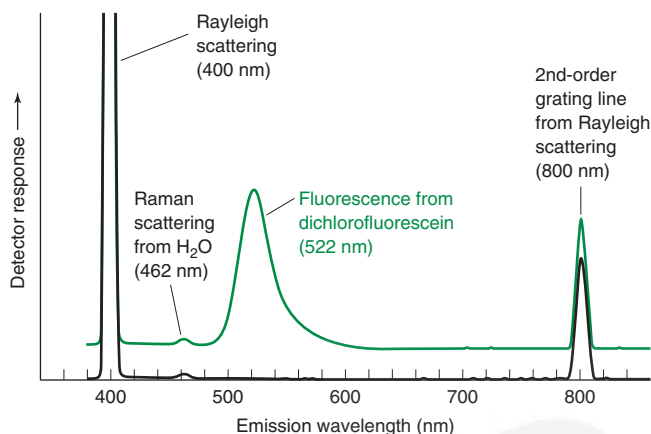
**Emission spectrum:** constant  $\lambda_{ex}$  and variable  $\lambda_{em}$

**Excitation spectrum:** variable  $\lambda_{ex}$  and constant  $\lambda_{em}$

Equation 18-8 comes from Equations 18-5 and 18-6. If species other than analyte absorbed at the wavelengths of interest, we would have to include their absorbance.

Molecule	Quantum yield <sup>a</sup>
Benzene	0.05
Anthracene	0.36
Quinine	0.55
Rhodamine 6G	0.95
Fluorescein	0.97

Emission spectra can exhibit confusing features in addition to fluorescence and phosphorescence. The emission spectrum of aqueous dichlorofluorescein (colored line) and an emission spectrum of pure water (black line) are shown below. The excitation wavelength is 400 nm. The only difference between the two traces is fluorescence from dichlorofluorescein with a peak at 522 nm.



Upper trace: Emission spectrum of aqueous dichlorofluorescein Lower trace: Spectrum observed from pure water [Information from Kris Varazo, Francis Marion University. See R. J. Clarke and A. Oprys, "Fluorescence and Light Scattering," *J. Chem. Ed.* **2004**, 81, 705.]

What are the other peaks? The strongest peak, which is off-scale, is observed at the excitation wavelength of 400 nm. It is called **Rayleigh scattering** after the same Lord Rayleigh (J. W. Strutt) who discovered argon (page 78). The oscillating electromagnetic field of the excitation light source causes electrons in water molecules to oscillate at the same frequency as the incident radiation. Oscillating electrons emit this same frequency of radiation in all directions. The time required for scattering is essentially the period of one oscillation of the incoming electromagnetic wave, which is  $\sim 10^{-15}$  s for 400-nm light. By comparison, the time for fluorescence is  $\sim 10^{-9}$  to  $10^{-4}$  s. Rayleigh scattering is always present and is usually filtered out so that it is not displayed in the emission spectrum.

The second strongest peak in this example comes at 800 nm, which is exactly twice the excitation wavelength. It is an artifact of the monochromator. Grating monochromators designed to pass wavelength  $\lambda$  also pass integer fractions  $\lambda/2$ ,  $\lambda/3$ , and so on, with decreasing efficiency. When the emission monochromator in Figure 18-22 is set to pass 800 nm, it also passes some light at 400 nm. Rayleigh scattering at 400 nm passes through the monochromator set to 800 nm. We call this *second-order diffraction* from the monochromator. If we used a filter to block 400-nm light between the sample cell and the emission monochromator in Figure 18-22, there would be no peak at 800 nm in the emission spectrum.

A weak but reproducible peak is observed in both water and dichlorofluorescein solution at 462 nm. The difference in energy between incident light at 400 nm and the peak at 462 nm corresponds to a vibrational energy of  $\text{H}_2\text{O}$ . The peak at 462 nm is called **Raman scattering** after the Indian physicist C. V. Raman, who discovered this phenomenon in 1928 and was awarded the Nobel Prize in 1930. In this type of scattering, which also occurs in a time frame of  $\sim 10^{-15}$  s, a small fraction of incident photons gives up one quantum of molecular vibrational energy to  $\text{H}_2\text{O}$ . Scattered radiation emerges with less energy than the excitation energy. Vibrational energy is customarily expressed as the wavenumber ( $\text{cm}^{-1}$ ) of a photon with that energy. Liquid  $\text{H}_2\text{O}$  has a broad range of vibrational energies centered near  $3\,404\text{ cm}^{-1}$ . The wavenumber of the exciting radiation is  $1/\text{wavelength} = 1/400\text{ nm} = 25\,000\text{ cm}^{-1}$ . In Raman scattering, an incident photon with energy of  $25\,000\text{ cm}^{-1}$  gives up  $3\,404\text{ cm}^{-1}$  and emerges at  $(25\,000 - 3\,404) = 21\,596\text{ cm}^{-1}$ . The wavelength is  $1/(21\,596\text{ cm}^{-1}) \approx 463\text{ nm}$ . The observed peak is at 462 nm.

What are the lessons from this example? First, compare the spectrum of pure solvent to the spectrum of a sample under study so that you can disregard peaks due to solvent. Second, fluorescence comes at a fixed location, such as 522 nm for dichlorofluorescein. The wavelength of scattered radiation varies with excitation wavelength. If we used an excitation wavelength of 410 nm instead of 400, the second-order grating line would be seen at 820 nm and the water Raman line would be at an energy that is  $3\,404\text{ cm}^{-1}$  less than the exciting light, or 477 nm. Scattered radiation shifts with the incident wavelength, but fluorescence and phosphorescence do not.

Combining Equations 18-10 and 18-11 gives an expression for emission intensity:

$$I = k'\Phi(P_0' - P')10^{-\epsilon_{\text{em}}b_3c}$$

Substituting expressions for  $P_0'$  and  $P'$  from Equations 18-8 and 18-9, we obtain a relation between incident irradiance and emission intensity:

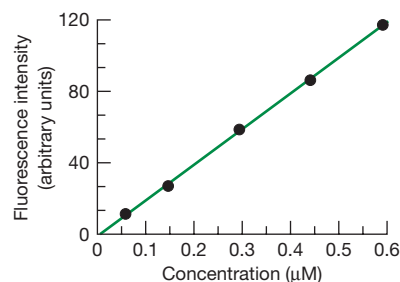
$$\begin{aligned} I &= k'\Phi(P_0 \cdot 10^{-\epsilon_{\text{ex}}b_1c} - P_0 \cdot 10^{-\epsilon_{\text{ex}}b_1c} \cdot 10^{-\epsilon_{\text{ex}}b_2c})10^{-\epsilon_{\text{em}}b_3c} \\ &= k'\Phi P_0 \cdot \underbrace{10^{-\epsilon_{\text{ex}}b_1c}}_{\text{Loss of intensity in region 1}} \underbrace{(1 - 10^{-\epsilon_{\text{ex}}b_2c})}_{\text{Absorption in region 2}} \underbrace{10^{-\epsilon_{\text{em}}b_3c}}_{\text{Loss of intensity in region 3}} \end{aligned} \quad (18-12)$$

Consider the limit of low concentration, which means that the exponents  $\epsilon_{\text{ex}}b_1c$ ,  $\epsilon_{\text{ex}}b_2c$ , and  $\epsilon_{\text{em}}b_3c$  are all very small. The terms  $10^{-\epsilon_{\text{ex}}b_1c}$ ,  $10^{-\epsilon_{\text{ex}}b_2c}$ , and  $10^{-\epsilon_{\text{em}}b_3c}$  are all close to unity. We can replace  $10^{-\epsilon_{\text{ex}}b_1c}$  and  $10^{-\epsilon_{\text{em}}b_3c}$  with 1 in Equation 18-12. We cannot replace  $10^{-\epsilon_{\text{ex}}b_2c}$  with 1 because we are subtracting this term from 1 and would be left with 0. Instead, we expand  $10^{-\epsilon_{\text{ex}}b_2c}$  in a power series:

$$10^{-\epsilon_{\text{ex}}b_2c} = 1 - \epsilon_{\text{ex}}b_2c \ln 10 + \frac{(\epsilon_{\text{ex}}b_2c \ln 10)^2}{2!} - \frac{(\epsilon_{\text{ex}}b_2c \ln 10)^3}{3!} + \dots \quad (18-13)$$

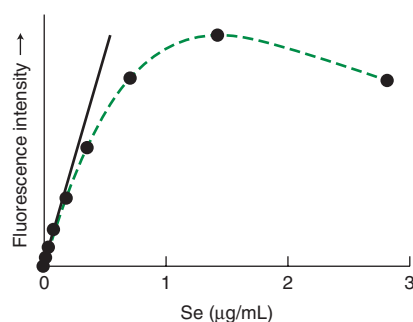
The series 18-13 follows from the relation  $10^{-A} = (e^{\ln 10})^{-A} = e^{-A \ln 10}$  and the expansion of  $e^x$ :

$$e^x = 1 + \frac{x^1}{1!} + \frac{x^2}{2!} + \frac{x^3}{3!} + \dots$$



**FIGURE 18-24** Linear calibration curve for fluorescence of anthracene measured at the wavelength of maximum fluorescence in Figure 18-23. [Data from C. M. Byron and T. C. Werner, "Experiments in Synchronous Fluorescence Spectroscopy for the Undergraduate Instrumental Chemistry Course," *J. Chem. Ed.* **1991**, 68, 433.]

**Derivatization** is the chemical alteration of analyte so that it can be detected easily or separated easily from other species.



**FIGURE 18-25** Calibration curve for fluorescent product in Reaction 18-15. Calibration is linear only below 0.1 μg/mL Se. Curvature and maximum are due to self-absorption. [Data from M.-C. Sheffield and T. M. Nahir, "Analysis of Selenium in Brazil Nuts by Microwave Digestion and Fluorescence Detection," *J. Chem. Ed.* **2002**, 79, 1345.]

Each term of 18-13 becomes smaller and smaller, so we just keep the first two terms. The central factor in Equation 18-12 becomes  $(1 - 10^{-\epsilon_{\text{ex}} b_2 c}) = (1 - [1 - \epsilon_{\text{ex}} b_2 c \ln 10]) = \epsilon_{\text{ex}} b_2 c \ln 10$ , so the entire equation can be written

$$\text{Emission intensity at low concentration: } k' \Phi P_0 (\epsilon_{\text{ex}} b_2 c \ln 10) = I = k \Phi P_0 c \quad (18-14)$$

where  $k = k' \epsilon_{\text{ex}} b_2 \ln 10$  is a constant.

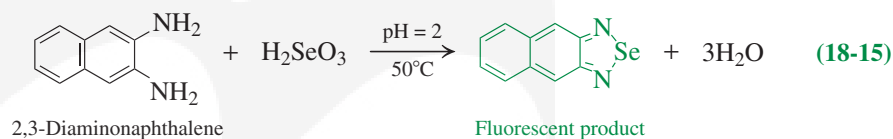
Equation 18-14 says that, *at low concentration, emission intensity is proportional to analyte concentration*. Data for anthracene in Figure 18-24 are linear below  $10^{-6}$  M. Blank samples invariably scatter light and must be run in every analysis. Equation 18-14 tells us that doubling the incident irradiance ( $P_0$ ) will double the emission intensity (up to a point). In contrast, doubling  $P_0$  has no effect on absorbance, which is a *ratio* of two intensities.

For higher concentrations, we need all the terms in Equation 18-12, or we need an even more accurate equation or correction techniques.<sup>23</sup> As concentration increases, maximum emission is reached. Then emission decreases because absorption increases more rapidly than the emission. We say the emission is **quenched** (decreased) by **self-absorption**, which is the absorption of excitation or emission irradiance by analyte molecules in the solution. Quenching by self-absorption is also called the *inner filter effect*. At high concentration, even the *shape* of the emission spectrum can change because absorption and emission both depend on wavelength. Absorbance at excitation and emission wavelengths should be <0.1 to minimize inner filter effects. Fluorescence may also be quenched by matrix components or oxygen from the air. Matrix-matched standards or standard addition calibration may be needed.

### Example: Fluorimetric Assay of Selenium in Brazil Nuts

Selenium is a trace element essential to life. For example, the selenium-containing enzyme glutathione peroxidase catalyzes the destruction of peroxides (ROOH) that are harmful to cells. Conversely, at high concentration, selenium can be toxic.

To measure selenium in Brazil nuts, 0.1 g of nut is digested with 2.5 mL of 70 wt% HNO<sub>3</sub> in a Teflon *bomb* (Figure 28-7) in a microwave oven. Hydrogen selenate (H<sub>2</sub>SeO<sub>4</sub>) in the digest is reduced to hydrogen selenite (H<sub>2</sub>SeO<sub>3</sub>) with hydroxylamine (NH<sub>2</sub>OH). Selenite is then **derivatized** to form a fluorescent product that is extracted into cyclohexane.



Maximum response of the fluorescent product was observed with an excitation wavelength of 378 nm and an emission wavelength of 518 nm. In Figure 18-25, emission is proportional to concentration only up to ~0.1 μg Se/mL. Beyond 0.1 μg Se/mL, the response becomes curved, eventually reaches a maximum, and finally *decreases* with increasing concentration as self-absorption dominates. This behavior is predicted by Equation 18-12.

## Terms to Understand

absorbance	excited state	monochromatic light	rotational transition
absorption spectrum	fluorescence	monochromator	self-absorption
Beer's law	frequency	phosphorescence	singlet state
chromophore	ground state	photon	spectrophotometric titration
cuvette	hertz	quantum yield	spectrophotometry
derivatization	irradiance	quenching	transmittance
electromagnetic spectrum	luminescence	Raman scattering	triplet state
electronic transition	masking	Rayleigh scattering	vibrational transition
emission spectrum	molar absorptivity	reagent blank	wavelength
excitation spectrum	molecular orbital	refractive index	wavenumber



## Summary

Light can be thought of as waves whose wavelength ( $\lambda$ ) and frequency ( $\nu$ ) have the important relation  $\lambda\nu = c$ , where  $c$  is the speed of light. Alternatively, light may be viewed as consisting of photons whose energy ( $E$ ) is given by  $E = h\nu = hc/\lambda = hc\tilde{\nu}$ , where  $h$  is Planck's constant and  $\tilde{\nu}$  ( $= 1/\lambda$ ) is the wavenumber. Absorption of light is commonly measured by absorbance ( $A$ ) or transmittance ( $T$ ), defined as  $A = \log(P_0/P)$  and  $T = P/P_0$ , where  $P_0$  is the incident irradiance and  $P$  is the exiting irradiance. Absorption spectroscopy is useful in quantitative analysis because absorbance is proportional to the concentration of the absorbing species in dilute solution (Beer's law):  $A = \epsilon bc$ . In this equation,  $b$  is pathlength,  $c$  is concentration, and  $\epsilon$  is the molar absorptivity (a constant of proportionality). You should be able to prepare a series of standard solutions for calibration by serial dilution of a concentrated standard.

Basic components of a spectrophotometer include a radiation (light) source, a monochromator, a sample cell, and a detector. To minimize errors in spectrophotometry, standards and samples must be prepared using reproducible technique, solutions should be free of particles, cuvettes must be clean, and they should be

positioned reproducibly in the sample holder. Measurements should be made at a wavelength of maximum absorbance. Instrument errors tend to be minimized if the absorbance falls in the range  $A \approx 0.3-2$ .

In a spectrophotometric titration, absorbance is monitored as titrant is added. For many reactions, there is an abrupt change in slope when the equivalence point is reached.

When a molecule absorbs light, it is promoted to an excited state from which it returns to the ground state by nonradiative processes or by fluorescence (singlet  $\rightarrow$  singlet emission) or phosphorescence (triplet  $\rightarrow$  singlet emission). Emission intensity is proportional to concentration at low concentrations. At sufficiently high concentration, emission decreases because of self-absorption by the analyte. An excitation spectrum (a graph of emission intensity versus excitation wavelength) is similar to an absorption spectrum (a graph of absorbance versus wavelength). An emission spectrum (a graph of emission intensity versus emission wavelength) is observed at lower energy than the absorption spectrum and tends to be the mirror image of the absorption spectrum.

## Exercises

**18-A. (a)** What value of absorbance corresponds to 45.0%  $T$ ?

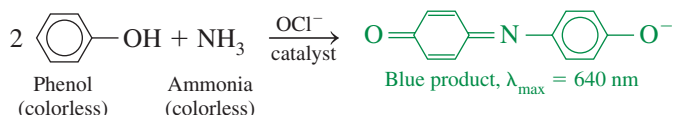
**(b)** If a 0.010 0 M solution exhibits 45.0%  $T$  at some wavelength, what will be the percent transmittance for a 0.020 0 M solution of the same substance?

**18-B. (a)** A  $3.96 \times 10^{-4}$  M solution of compound A exhibited an absorbance of 0.624 at 238 nm in a 1.000-cm quartz cuvette; a blank solution containing only solvent had an absorbance of 0.029 at the same wavelength. Find the molar absorptivity of compound A.

**(b)** The absorbance of an unknown solution of compound A in the same solvent and cuvette was 0.375 at 238 nm. Find the concentration of A in the unknown.

**(c)** A concentrated solution of compound A in the same solvent was diluted from an initial volume of 2.00 mL to a final volume of 25.00 mL and then had an absorbance of 0.733 in the same cuvette. What is the concentration of A in the concentrated solution?

**18-C.** In U.S. Environmental Protection Agency method 350.1, ammonia is determined spectrophotometrically by reaction with phenol in the presence of hypochlorite ( $\text{OCl}^-$ ) and nitroprusside  $[\text{Fe}(\text{CN})_5(\text{NO})]^{2-}$  catalyst:



To a 10.0-mL sample of surface water were added 1.00 mL phenol solution, 1.00 mL sodium nitroprusside solution, and 2.5 mL hypochlorite solution, with thorough mixing after each addition. The sample was diluted to 50.0 mL, thoroughly mixed, allowed to react for 1 hour, and then absorbance at 640 nm was measured in a 1.000-cm glass cuvette. In method 350.1, ammonia concentrations are quoted in mass of nitrogen (N) per volume. A 100.0 mg N/L stock solution was prepared from 0.381 9 g of  $\text{NH}_4\text{Cl}$  (FM 53.49) dissolved in 1.00 L of water. Working standards of 0.100 to 1.00 mg N/L were prepared by dilution of the stock solution. Then 10.0 mL of each standard were diluted in a 50-mL volumetric flask and

analyzed in the same manner as the unknown. A reagent blank was prepared by using distilled water in place of unknown.

Standard (mg N/L)	Absorbance at 640 nm
Blank	0.028
0.100	0.089
0.300	0.200
0.500	0.306
0.700	0.428
1.00	0.597

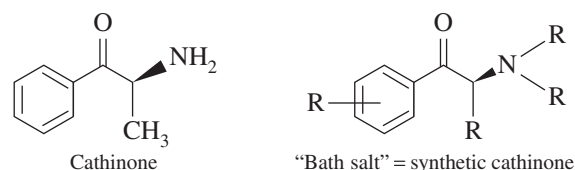
**(a)** Calculate the molar absorptivity of the blue product using the 0.500 mg N/L standard.

**(b)** Plot a calibration curve of corrected absorbance versus ammonia concentration. Is the calibration linear based on visual inspection? *Hint:* Residuals—the vertical distance between data points and line—should be randomly distributed about the line (page 106).

**(c)** Calculate the concentration of ammonia in mg N/L for an unknown with  $A = 0.272$ .

**(d)** How can you make the ammonia calibration standards from the 100.0 mg N/L stock solution using transfer pipets and volumetric flasks?

**18-D.** Cathinone is the psychoactive alkaloid in khat leaves that grow on the Arabian Peninsula. Some “bath salts” are synthetic variants of cathinone designed to circumvent laws, but with unknown properties and side effects.



*Presumptive tests* are used in forensics to establish whether a sample either does not contain or potentially may contain a particular substance.



A presumptive test for synthetic cathinones is based on reduction of  $\text{Cu}^{2+}$  to  $\text{Cu}^+$  by a cathinone in the presence of neocuproine.<sup>24</sup> If cathinones are present, and other reducing compounds are absent, the colored complex  $\text{Cu}(\text{neocuproine})_2^+$  is formed, with an absorption maximum at 454 nm. A spectrophotometric procedure for analysis of cathinone in khat leaves is:

1. *Solution of khat extract:* Cut dried khat leaves into small pieces and extract with ethanol. Filter and quantitatively transfer extract to a 500-mL volumetric flask. Bring to volume with distilled water and thoroughly mix.
2. Transfer 1.00 mL of solution from the 500-mL flask to a 25-mL volumetric flask and add  $\text{Cu}^{2+}$  solution. Then add 1.00 mL of neocuproine and 10 mL of distilled water and briefly boil.
3. After cooling, add 1.25 mL of acetate buffer so the pH is suitable for complex formation, and dilute to 25 mL with distilled water.
4. Measure absorbance of the yellow  $\text{Cu}(\text{neocuproine})_2^+$  at 454 nm in a 1.000-cm glass cuvette.

(a) *External calibration:* Cathinone standards (1.00 mL containing 2.5 to 10.0  $\mu\text{g/mL}$ ) and 1.00 mL of khat extract from 1.27 g of dried leaves were analyzed. What is the concentration of cathinone in the extract? From the absorbance data below, what was the concentration of cathinone ( $\mu\text{g/g}$ ) in the dried khat leaves?

Cathinone ( $\mu\text{g/mL}$ )	Absorbance
Blank	0.03
2.5	0.20
5.0	0.37
7.5	0.52
10.0	0.71
Khat leaves extract	0.59

SOURCE: Data from A. M. Al-Obaid, S. A. Al-Tamrah, F. A. Aly, and A. A. Alwarthan, "Determination of (S)(-)-Cathinone by Spectrophotometric Detection," J. Pharm. Biomed. Anal. **1998**, 17, 321.

(b) *Standard addition:* Solutions containing 1.0 mL of extract from 1.12 g of dried khat and increasing amounts of cathinone were prepared. What was the concentration of cathinone ( $\mu\text{g/g}$ ) in the dried khat leaves?

Cathinone added ( $\mu\text{g}$ )	Absorbance
Blank	0.029
0	no data
4.0	0.800
8.0	1.090
12.0	1.359
16.0	1.637

**18-E.** Semi-xylanol orange is a yellow compound at pH 5.9 but turns red when it reacts with  $\text{Pb}^{2+}$ . A 2.025-mL sample of semi-xylanol orange at pH 5.9 was titrated with  $7.515 \times 10^{-4}$  M  $\text{Pb}(\text{NO}_3)_2$ , with the following results:

Total $\mu\text{L}$ $\text{Pb}^{2+}$ added	Absorbance at 490-nm wavelength	Total $\mu\text{L}$ $\text{Pb}^{2+}$ added	Absorbance at 490-nm wavelength
0.0	0.227	42.0	0.425
6.0	0.256	48.0	0.445
12.0	0.286	54.0	0.448
18.0	0.316	60.0	0.449
24.0	0.345	70.0	0.450
30.0	0.370	80.0	0.447
36.0	0.399		

Make a graph of absorbance versus microliters of  $\text{Pb}^{2+}$  added. Be sure to correct the absorbances for dilution. Corrected absorbance is what would be observed if the volume were not changed from its initial value of 2.025 mL. Assuming that the reaction of semi-xylanol orange with  $\text{Pb}^{2+}$  has a 1:1 stoichiometry, find the molarity of semi-xylanol orange in the original solution.

## Problems

### Properties of Light

**18-1.** Fill in the blanks.

- (a) If you double the frequency of electromagnetic radiation, you \_\_\_\_\_ the energy.
- (b) If you double the wavelength, you \_\_\_\_\_ the energy.
- (c) If you double the wavenumber, you \_\_\_\_\_ the energy.

**18-2. (a)** How much energy (in kilojoules) is carried by one mole of photons of red light with  $\lambda = 650$  nm?

(b) How many kilojoules are carried by one mole of photons of violet light with  $\lambda = 400$  nm?

**18-3. (a)** Calculate the frequency (Hz), wavenumber ( $\text{cm}^{-1}$ ), and energy (J/photon and J/[mol of photons]) of visible light with a wavelength of 562 nm.

(b) Calculate the wavelength ( $\mu\text{m}$ ), frequency (Hz), and energy (J/photon and J/[mol of photons]) of infrared radiation with a wavenumber of  $3\,028\text{ cm}^{-1}$ .

**18-4.** Which molecular processes correspond to the energies of microwave, infrared, visible, and ultraviolet photons?

**18-5.** Characteristic orange light produced by sodium in a flame is due to an intense emission called the sodium D line, which is actually a doublet, with wavelengths (measured in vacuum) of 589.158 and

589.756 nm. The index of refraction of dry air ( $15^\circ\text{C}$ , 101.325 kPa, 450 ppm  $\text{CO}_2$ ) at 589 nm is 1.000 277. Calculate the frequency, wavelength, and wavenumber of each component of the D line, measured in air.

### Absorption of Light and Measuring Absorbance

**18-6.** How do transmittance, absorbance, and molar absorptivity differ? Which one is proportional to concentration?

**18-7.** What is an absorption spectrum?

**18-8.** Why does a compound whose visible absorption maximum is at 480 nm (blue-green) appear to be red?

**18-9. Color and absorption spectra.** Color Plate 17 shows colored solutions and their spectra. From Table 18-1, state what color is absorbed and predict the color of each solution from the wavelength of maximum absorption. Do observed colors agree with predicted colors?

**18-10.** Why is it most accurate to measure absorbances in the range  $A = 0.3 - 2$ ?

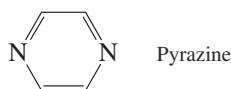
**18-11.** The absorbance of a  $2.31 \times 10^{-5}$  M solution of a compound is 0.822 at a wavelength of 266 nm in a 1.000-cm cell.

- (a) Calculate the molar absorptivity at 266 nm.
- (b) What material(s) should the cuvette be made from?

**18-12.** What color would you expect to observe for a solution of  $\text{Fe(ferrozine)}_3^{4-}$ , which has a visible absorbance maximum at 562 nm? Of what material(s) should the cuvette be constructed?

**18-13.** When I was a boy, Uncle Wilbur let me watch as he analyzed the iron content of runoff from his banana ranch. A 25.0-mL sample was acidified with nitric acid and treated with excess KSCN to form a red complex. (KSCN itself is colorless.) The solution was then diluted to 100.0 mL and put in a variable-pathlength cell. For comparison, a 10.0-mL reference sample of  $6.80 \times 10^{-4} \text{ M Fe}^{3+}$  was treated with  $\text{HNO}_3$  and KSCN and diluted to 50.0 mL. The reference was placed in a cell with a 1.00-cm light path. The runoff sample exhibited the same absorbance as the reference when the pathlength of the runoff cell was 2.48 cm. What was the concentration of iron in Uncle Wilbur's runoff?

**18-14.** Solid pyrazine has a vapor pressure of 30.3  $\mu\text{bar}$  and a transmittance of 24.4% at a wavelength of 266 nm in a 3.00-cm cell at 298 K.



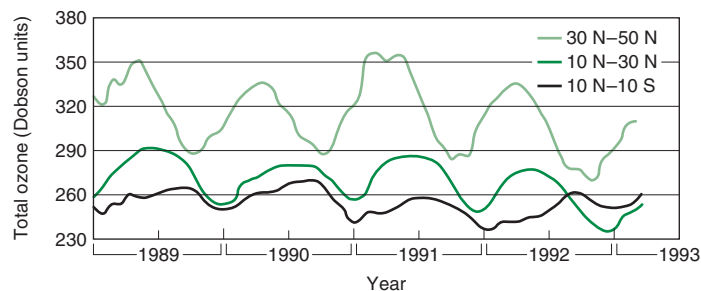
- (a) Convert transmittance to absorbance.
- (b) Convert pressure to concentration (mol/L) using the ideal gas law (Problem 1-17).
- (c) Find the molar absorptivity of gaseous pyrazine at 266 nm.

**18-15.** The *absorption cross section* on the ordinate of the ozone absorption spectrum at the beginning of this chapter is defined by

$$\text{Transmittance } (T) = e^{-n\sigma b}$$

where  $n$  is the number of absorbing molecules per cubic centimeter,  $\sigma$  is the absorption cross section ( $\text{cm}^2$ ), and  $b$  is the pathlength (cm). The total ozone in the atmosphere is approximately  $8 \times 10^{18}$  molecules above each square centimeter of Earth's surface (from the surface up to the top of the atmosphere). If this were compressed into a 1-cm-thick layer, the concentration would be  $8 \times 10^{18}$  molecules/ $\text{cm}^3$ .

- (a) Ultraviolet radiation in the 200 to 280 nm range is essentially completely blocked by ozone in the atmosphere but barely absorbed in the 315 to 400 nm range. Using the ozone spectrum in the Chapter 18 opener, estimate the transmittance and absorbance of this 1-cm<sup>3</sup> sample at 280 and at 340 nm.
- (b) Sunburns are caused by radiation in the 295- to 310-nm region. At the center of this region, the transmittance of atmospheric ozone is 0.14. Calculate the absorption cross section for  $T = 0.14$ ,  $n = 8 \times 10^{18}$  molecules/ $\text{cm}^3$ , and  $b = 1$  cm. By what percentage does the transmittance increase if the ozone concentration decreases by 1% to  $7.92 \times 10^{18}$  molecules/ $\text{cm}^3$ ?
- (c) Atmospheric  $\text{O}_3$  is measured in *Dobson units* (1 unit =  $2.69 \times 10^{16}$  molecules  $\text{O}_3$  above each  $\text{cm}^2$  of Earth's surface). (Dobson unit  $\equiv$  thickness [in hundredths of a millimeter] that the  $\text{O}_3$  column would occupy if it were compressed to 1 atm at 0°C.) The graph shows variations in  $\text{O}_3$  concentration as a function of latitude and season. Using an absorption cross section of  $2.5 \times 10^{-19} \text{ cm}^2$ , calculate the transmittance in the winter and in the summer at 30°–50° N latitude, at which  $\text{O}_3$  varies from 290 to 350 Dobson units. By what percentage is the ultraviolet transmittance greater in winter than in summer?



Variation in atmospheric ozone at different latitudes [Data from P. S. Zurer, *Chem. Eng. News*, 24 May 1993, p. 8.]

## Beer's Law in Chemical Analysis

**18-16.** For serum iron analysis using ferrozine in Section 18-4:

- (a) What would the calibration curve look like if the reagent blank absorbance of 0.038 were not subtracted from absorbance readings?
- (b) What would the blank-corrected calibration curve look like if absorbance were measured at 600 nm rather than 562 nm? Why is it poor practice to measure absorbance on the steep side of an absorption peak rather than at the peak wavelength?
- (c) What should you do if the blank-corrected absorbance of the unknown is 1.246? *Hint:* Look at range in Figure 18-9.

**18-17. Quality assurance.** For serum iron analysis using ferrozine in Section 18-4:

- (a) What is the purpose of analyzing a certified reference material after the method was developed? What is a certified reference material?
- (b) The reference material was certified to contain 1.36  $\mu\text{g Fe/mL}$ . Five replicate analyses using ferrozine without neocuproine yielded a mean of 1.49<sub>3</sub>  $\mu\text{g Fe/mL}$  with a standard deviation of 0.04<sub>5</sub>  $\mu\text{g Fe/mL}$ . Does the analysis agree with the true value within the 95% confidence interval?
- (c) What is the purpose of neocuproine in the serum iron analysis?
- (d) Four replicate analyses using ferrozine with neocuproine yielded 1.38<sub>7</sub>  $\mu\text{g} \pm 0.04_3 \text{ Fe/mL}$ . Does addition of neocuproine make a statistically significant difference in the result from (b)?

**18-18.** A compound with molecular mass 292.16 g/mol was dissolved in a 5-mL volumetric flask. A 1.00-mL aliquot was withdrawn, placed in a 10-mL volumetric flask, and diluted to the mark. The absorbance at 340 nm was 0.427 in a 1.000-cm cuvette. The molar absorptivity at 340 nm is  $\epsilon_{340} = 6130 \text{ M}^{-1} \text{ cm}^{-1}$ .


- (a) Calculate the concentration of the compound in the cuvette.
- (b) What was the concentration of the compound in the 5-mL flask?
- (c) How many milligrams of compound were used to make the 5-mL solution?

**18-19.** If a sample for spectrophotometric analysis is placed in a 10-cm cell, the absorbance will be 10 times greater than the absorbance in a 1-cm cell. Will the absorbance of the reagent-blank solution also be increased by a factor of 10?

**18-20.** You have been sent to India to investigate the occurrence of goiter disease attributed to iodine deficiency. As part of your investigation, you must make field measurements of traces of iodide ( $\text{I}^-$ ) in groundwater. The procedure is to oxidize  $\text{I}^-$  to  $\text{I}_2$  and convert the  $\text{I}_2$  into an intensely colored complex with the dye brilliant green in the organic solvent toluene.

(a) A  $3.15 \times 10^{-6}$  M solution of the colored complex exhibited an absorbance of 0.267 at 635 nm in a 1.000-cm cuvette. A blank solution made from distilled water in place of groundwater had an absorbance of 0.019. Find the molar absorptivity of the colored complex.

(b) The absorbance of an unknown solution prepared from groundwater was 0.175. Find the concentration of the unknown.

**18-21.**  **DNA concentration.** The nucleotide bases of DNA and RNA are aromatic and absorb ultraviolet light (Appendix L). The concentration of DNA or RNA can be determined by monitoring absorbance at 260 nm. Calibration data for a 15-base single-stranded DNA (4 568 g/mol) in a 1-cm cell are shown in the table.

DNA ( $\mu\text{g/mL}$ )	Absorbance	DNA ( $\mu\text{g/mL}$ )	Absorbance
2.5	0.080	15.0	0.442
5.0	0.149	20.0	0.620
10.0	0.307	25.0	0.739

SOURCE: Data from S.-H. Li, A. Jain, T. Tscharnke, T. Arnold, and D. W. Trau, "Hand-Held Photometer for Instant On-Spot Quantification of Nucleic Acids, Proteins, and Cells," *Anal. Chem.* **2018**, 90, 2564.

(a) Use Excel to draw a graph of the calibration data and add a trend-line. Write the equation for the line including standard uncertainties in the slope and intercept. Is the calibration linear? *Hint:* Residuals—the vertical distance between data points and line—should be randomly distributed about the line (page 106).

(b) What is the molar absorptivity of the single-stranded DNA in (a)?

(c) The absorptivity of DNA and RNA depends on the nucleotides present and their sequence.<sup>13</sup> A popular online encyclopedia says absorptivity for *all* single-stranded DNA is  $0.027 (\mu\text{g/mL})^{-1}\text{cm}^{-1}$ . How much error would result if the Web site value were used for this DNA sample?

(d) Analysis of a DNA sample in triplicate with a hand-held photometer yielded a mean concentration of  $8.264 \mu\text{g/mL}$  and a standard deviation of  $0.017 \mu\text{g/mL}$ . A commercial spectrometer gave  $8.385 \pm 0.154 \mu\text{g/mL}$  for  $n = 3$ . Are the precisions statistically equivalent? Are the concentrations statistically equivalent?

**18-22. Protein concentration.** The molar absorptivity of a protein in water at 280 nm can be estimated within  $\sim 5\text{--}10\%$  from its content of the amino acids tyrosine and tryptophan (Table 10-1) and from the number of disulfide linkages (R–S–S–R) between cysteine residues.<sup>14</sup>

$$\epsilon_{280 \text{ nm}} (\text{M}^{-1} \text{ cm}^{-1}) \approx 5\,500 n_{\text{Tyr}} + 1\,490 n_{\text{Trp}} + 125 n_{\text{S-S}}$$

where  $n_{\text{Tyr}}$  is the number of tryptophans,  $n_{\text{Trp}}$  is the number of tyrosines, and  $n_{\text{S-S}}$  is the number of disulfides. The protein human serum transferrin has 679 amino acids including 8 tryptophans, 26 tyrosines, 19 disulfide linkages, and 2 carbohydrate chains of variable length.<sup>25</sup> The molecular mass with the most common carbohydrates is 79 570.

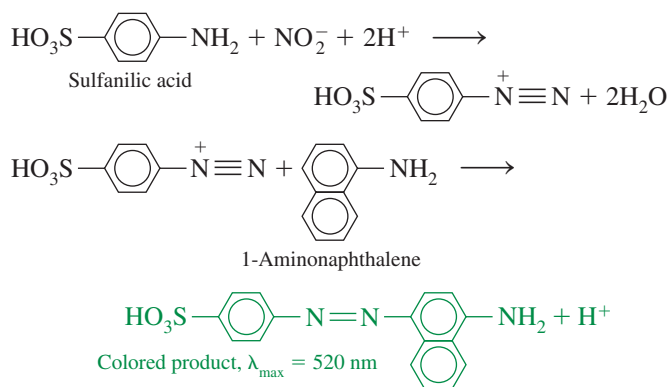
(a) Predict the molar absorptivity of transferrin.

(b) Predict the absorbance of a 1 wt% transferrin solution in a 1.000-cm-pathlength cuvette. *Hint:* The value tabulated by Pace et al.<sup>14</sup> is 11.10.

(c) From your answer to (a), estimate wt% and mg/mL of a transferrin solution with an absorbance of 1.50 at 280 nm.

**18-23.** Nitrite ion,  $\text{NO}_2^-$ , is a preservative for bacon and other foods, but it is potentially carcinogenic. AOAC standard method

973.31 for  $\text{NO}_2^-$  in cured meat is based on the Griess test, which uses the reactions:



Here is an abbreviated procedure for the determination:

- To 50.0 mL of an unknown solution containing nitrite is added 1.00 mL of sulfanilic acid solution.
- After 10 min, 2.00 mL of 1-aminonaphthalene solution and 1.00 mL of buffer are added.
- After 15 min, the absorbance is read at 520 nm in a 5.00-cm cell. The following solutions were analyzed:

- 50.0 mL of food extract known to contain no nitrite (that is, a negligible amount); final absorbance = 0.153.
- 50.0 mL of food extract suspected to contain nitrite; final absorbance = 0.622.
- Same as B, but fortified with  $10.0 \mu\text{L}$  of  $7.50 \times 10^{-3}$  M  $\text{NaNO}_2$  added to the 50.0-mL sample; final absorbance = 0.967.

(a) Calculate the molar absorptivity,  $\epsilon$ , of the colored product. Remember that a 5.00-cm cell was used.

(b) How many micrograms of  $\text{NO}_2^-$  were present in 50.0 mL of food extract?

(c) Parts per million of nitrite may be expressed based on the mass of  $\text{NO}_2^-$  or on the mass of nitrogen within nitrite ( $\text{NO}_2^-$  nitrogen). Determine the mg/L of nitrite in the 50.0 mL food extract using both formats.

**18-24. Preparing standards for a calibration curve.**

(a) How much  $\text{Fe}(\text{H}_3\text{NCH}_2\text{CH}_2\text{NH}_3)(\text{SO}_4)_2 \cdot 4\text{H}_2\text{O}$  (ferrous ethylenediammonium sulfate, FM 382.13) should be dissolved in a 500-mL volumetric flask with 1 M  $\text{H}_2\text{SO}_4$  to obtain a stock solution with  $\sim 500 \mu\text{g Fe/mL}$ ?

(b) When making stock solution (a), you actually weigh out 1.627 g of reagent. What is the Fe concentration in  $\mu\text{g Fe/mL}$ ?

(c) How would you prepare 500 mL of standard containing  $\sim 1, 2, 3, 4,$  and  $5 \mu\text{g Fe/mL}$  in 0.1 M  $\text{H}_2\text{SO}_4$  from stock solution (b) using any Class A pipets from Table 2-4 with only 500-mL volumetric flasks?

(d) To reduce the generation of chemical waste, describe how you could prepare 50 mL of standard containing  $\sim 1, 2, 3, 4,$  and  $5 \mu\text{g Fe/mL}$  in 0.1 M  $\text{H}_2\text{SO}_4$  from stock solution (b) by serial dilution using any Class A pipets from Table 2-4 with only 50-mL volumetric flasks?

**18-25. Preparing standards for a calibration curve.**

(a) How much ferrous ammonium sulfate ( $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2 \cdot 6\text{H}_2\text{O}$ , FM 392.12) should be dissolved in a 500-mL volumetric flask with 1 M  $\text{H}_2\text{SO}_4$  to obtain a stock solution with 1 000  $\mu\text{g Fe/mL}$ ?

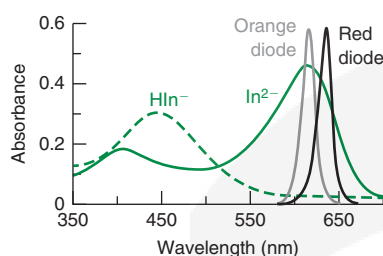


(b) When making stock solution (a), you weighed out 3.627 g of reagent. What is the Fe concentration in  $\mu\text{g Fe/mL}$ ?

(c) How would you prepare 250 mL of standard containing ~1, 2, 3, 4, 5, 7, 8, and 10 ( $\pm 20\%$ )  $\mu\text{g Fe/mL}$  in 0.1 M  $\text{H}_2\text{SO}_4$  from stock solution (b) using only 5- and 10-mL Class A pipets, only 250-mL volumetric flasks, and only two consecutive dilutions of the stock solution? For example, to prepare a solution with  $\sim 4 \mu\text{g Fe/mL}$  you could first dilute 15 mL ( $= 10 + 5 \text{ mL}$ ) of stock solution up to 250 mL to get  $\sim \left(\frac{15}{250}\right)(1000 \mu\text{g Fe/mL}) = \sim 60 \mu\text{g Fe/mL}$ . Then dilute 15 mL of the new solution up to 250 mL again to get  $\sim \left(\frac{15}{250}\right)(60 \mu\text{g Fe/mL}) = \sim 3.6 \mu\text{g Fe/mL}$ .

**18-26. Uncertainty in preparing standard solution.** Using uncertainties of Class A volumetric flasks and pipets in Tables 2-3 and 2-4, estimate the absolute and relative uncertainty (%) in the concentration of a solution made by diluting two 3-mL portions of stock solution containing  $1.086 \pm 0.002 \text{ g Fe/L}$  in 0.48 M HCl to 1 L in 0.1 M HCl.

**18-27.** A *photometer* is a simple spectrophotometer designed to monitor one or a few pre-determined wavelengths. Students at the University of British Columbia built a photometer using a colored light-emitting diode as their light source.



Absorption spectra for conjugate acid ( $\text{HIn}^-$ ) and base ( $\text{In}^{2-}$ ) forms of bromocresol green, and emission profiles of two colored light emitting diodes [Information from J. J. Wang, J. R. Rodríguez Núñez, E. J. Maxwell, and W. R. Algar, "Build Your Own Photometer: A Guided-Inquiry Experiment to Introduce Analytical Instrumentation," *J. Chem. Ed.* **2016**, 93, 166. J. J. Wang was an undergraduate researcher.]

(a) Which light-emitting diode would yield the greatest sensitivity (slope of the calibration curve) for the basic form ( $\text{In}^{2-}$ ) of bromocresol green?

(b) Which light-emitting diode would provide a more linear calibration plot? Why? If the light-emitting diode photometer fails to obey Beer's law, what would be the shape of the calibration curve?

(c) Below are calibration data for bromocresol green using a student-built light-emitting diode photometer and a commercial spectrometer at the same wavelength. Is the photometer calibration linear? *Hint:* How are data distributed about the line (page 106)?

Concentration ( $\mu\text{M}$ )	Absorbance spectrophotometer	Absorbance photometer
3.3	0.060	0.135
10.0	0.181	0.302
16.7	0.302	0.567
23.3	0.420	0.754
29.9	0.537	0.986

(d) The commercial spectrophotometer used a 1.000-cm cuvette. What is the pathlength of the student-built photometer?

### Spectrophotometric Titrations

**18-28.** A 100-mL sample of hard water containing magnesium and calcium was titrated as illustrated in Figure 18-10. It required 14.59 mL of 10.83 mM ethylenediaminetetraacetic acid (EDTA) to reach the end point.

(a) Why does the slope of the absorbance versus volume graph change abruptly at the equivalence point?

(b) How many moles of EDTA were required to reach the end point?

(c) Hardness of water is the total concentration of alkaline earth ions expressed as mg  $\text{CaCO}_3$  per liter as if all titrated cations were  $\text{CaCO}_3$ . What is the hardness of the 50-mL sample?

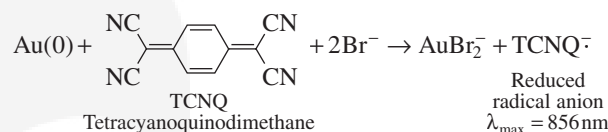
**18-29.** Use the spectra in Problem 18-27 to answer the following questions.

(a) Use the absorption maximum wavelength to predict the colors of the basic and acidic forms of bromocresol green.

(b) Sketch the approximate shape of the spectrophotometric titration curve for 30.0 mL of 0.00845 M HCl titrated with 0.049 6 M NaOH with bromocresol green indicator monitored at 610 nm. Indicate how the end point volume would be determined on your graph.

(c) What would be the shape of the spectrophotometric titration curves in (b) if absorbance is monitored at 445 nm? Which wavelength yields more precise results?

**18-30.** Gold nanoparticles (Figure 17-34) can be titrated with the oxidizing agent TCNQ in the presence of excess of  $\text{Br}^-$  to oxidize  $\text{Au}(0)$  to  $\text{AuBr}_2^-$  in deaerated toluene. Gold atoms in the interior of the particle are  $\text{Au}(0)$ . Gold atoms bound to  $\text{C}_{12}\text{H}_{25}\text{S}-$  (dodecanethiol) ligands on the surface of the particle are  $\text{Au}(I)$  and are not titrated.



Reduced  $\text{TCNQ}^-$  has a low-energy electronic absorption peak at 856 nm.

The table gives the absorbance at 856 nm when 0.700 mL of  $1.00 \times 10^{-4} \text{ M TCNQ} + 0.05 \text{ M } (\text{C}_8\text{H}_{17})_4\text{N}^+\text{Br}^-$  in toluene is titrated with gold nanoparticles (1.43 g/L in toluene) from a microsyringe with a Teflon-coated needle. Absorbance in the table has already been corrected for dilution.

Total $\mu\text{L}$ nanoparticles	Absorbance at 856 nm	Total $\mu\text{L}$ nanoparticles	Absorbance at 856 nm
4.9	0.208	19.1	0.706
8.0	0.301	22.0	0.770
11.0	0.405	25.0	0.784
13.7	0.502	30.0	0.785
16.2	0.610	35.9	0.784

Data from G. Zotti, B. Vercelli, and A. Berlin, "Reaction of Gold Nanoparticles with Tetracyanoquinoidal Molecules," *Anal. Chem.* **2008**, 80, 815.

(a) Make a graph of absorbance versus the volume of titrant and estimate the equivalence point. Calculate the mmol of  $\text{Au}(0)$  in 1.00 g of nanoparticles.

(b) Explain the shape of the titration curve.

(c) From other analyses of similarly prepared nanoparticles, it is estimated that 25 wt% of the mass of the particle is dodecanethiol ligand ( $\text{C}_{12}\text{H}_{25}\text{S}-$ , FM 201.39). Calculate mmol of  $\text{C}_{12}\text{H}_{25}\text{S}$  in 1.00 g of nanoparticles.

(d) The  $\text{Au}(I)$  content of 1.00 g of nanoparticles should be  $1.00 - \text{mass of Au}(0) - \text{mass of C}_{12}\text{H}_{25}\text{S}$ . Calculate the micromoles of  $\text{Au}(I)$  in 1.00 g of nanoparticles and the mole ratio  $\text{Au}(I) : \text{C}_{12}\text{H}_{25}\text{S}$ . In principle, this ratio should be 1 : 1. The difference is probably because wt%  $\text{C}_{12}\text{H}_{25}\text{S}$  was not measured for this specific nanoparticle preparation.

## Luminescence


**18-31.** In formaldehyde, the transition  $n \rightarrow \pi^*(T_1)$  occurs at 397 nm, and the  $n \rightarrow \pi^*(S_1)$  transition comes at 355 nm. What is the difference in energy (kJ/mol) between the  $S_1$  and  $T_1$  excited states? This difference is due to the different electron spins in the two states.


**18-32.** What is the difference between fluorescence and phosphorescence?

**18-33.** Explain what happens in Rayleigh scattering and in Raman scattering. How much faster is the scattering of visible light than fluorescence?

**18-34.** Consider a molecule that can fluoresce from the  $S_1$  state and phosphoresce from the  $T_1$  state. Which is emitted at longer wavelength, fluorescence or phosphorescence? Make a sketch showing absorption, fluorescence, and phosphorescence on a single spectrum.

**18-35.** What is the difference between a fluorescence excitation spectrum and a fluorescence emission spectrum? Which spectrum resembles an absorption spectrum?

**18-36.**  The wavelengths of maximum absorption and emission of anthracene in Figure 18-23 are approximately 357 and 402 nm. Molar absorptivities at these wavelengths are  $\epsilon_{\text{ex}} = 9.0 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$  and  $\epsilon_{\text{em}} = 0.05 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$ . Consider a fluorescence experiment in Figure 18-22 with cell dimensions  $b_1 = 0.30 \text{ cm}$ ,  $b_2 = 0.40 \text{ cm}$ , and  $b_3 = 0.50 \text{ cm}$ . Calculate the relative fluorescence intensity with Equation 18-12 as a function of concentration over the range  $10^{-8}$  to  $10^{-3} \text{ M}$ . Explain the shape of the curve. Up to approximately what concentration is fluorescence proportional to concentration (within 5%)? Is the calibration range in Figure 18-24 sensible?

**18-37.**  *Standard addition.* Selenium from 0.108 g of Brazil nuts was converted into the fluorescent product in Reaction 18-15, and extracted into 10.0 mL of cyclohexane. Then 2.00 mL of the cyclohexane solution were placed in a cuvette for fluorescence measurement. Standard additions of fluorescent product containing  $1.40 \mu\text{g Se/mL}$  are given in the table. Construct a standard addition graph such as Figure 5-7 to find the concentration of Se in the 2.00-mL unknown solution. Find the wt% of Se in the nuts and its uncertainty and 95% confidence interval.

Volume of standard added ( $\mu\text{L}$ )	Fluorescence intensity (arbitrary units)
0	41.4
10.0	49.2
20.0	56.4
30.0	63.8
40.0	70.3

**18-38.** *Literature problem.* Vanadium alloys containing 3–5% chromium and titanium are promising structural materials for fusion reactors. Mechanical and physical properties of the alloy depend on the concentrations of alloy elements. L. F. Tian, D. S. Zou, Y. C. Dai, L. L. Wang, and W. Gao in *Anal. Methods* **2017**, 9, 4471–4475 propose oxidation of chromium to chromate, followed by spectrophotometric measurement of chromate for the determination of chromium in such alloys.

(a) **Abstracts** provide quantitative information about the performance of the method. What is the range and precision of the method?

(b) **Introductions** explain the need for the new procedure. What alternate methods could be used for quantitation of chromium in vanadium-based alloys?

(c) **Experimental** describes the apparatus and methods. How was the solid alloy converted into a form suitable for spectrophotometric analysis?

(d) **Results and discussion** detail optimization of the steps in the procedure. What was the purpose of adding tartrate?

(e) Which wavelengths could have been used? Which was used? Why? *Hint:* Figures and tables contain key information.

(f) How was the accuracy of the method validated? *Hint:* Review page 107 for methods to validate accuracy.

**18-39.** *Literature problem.* The development of antibody-functionalized gold nanoparticles as reagents for spectrophotometric methods is an active area of research. The sensitivity of these methods depends on the amount of antibody on the nanoparticle. In *Analyst* **2016**, 141, 3851–3857, S. L. Filbrun and J. D. Driskell describe a fluorescence method that directly quantifies the amount of antibody immobilized on gold nanoparticles.

(a) How many antibodies adsorb onto each 60-nm gold nanoparticle? How much would the conventional method overestimate the antibody surface coverage?

(b) What alternate methods could be used for quantitation of the surface coverage of protein conjugated onto gold nanoparticles?

(c) What fluorescent reagent was used? What were the excitation and emission wavelengths?

(d) Is the fluorescence calibration linear? *Hint:* Inspect scatter of data about the line (page 106).

(e) What statistical test was used to compare the fluorescence method with an alternate method? *Hint:* Review Section 4-4 for types of tests that could be used.

(f) What is the y-intercept of the calibration curve for the absorbance assay using visible absorbance? *Hint:* Additional information such as calibration curves and spectra may be provided in the Electronic Supplementary Information that accompanies the paper on the journal Web site.