

国外化学名著系列

(影印版)1

〔美〕 William W. Parson

Modern Optical Spectroscopy

With Examples from Biophysics and Biochemistry

现代光谱学

——生物物理学与生物化学例析



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Preface

This book began as lecture notes for a course on optical spectroscopy that I taught for graduate students in biochemistry, chemistry, and our interdisciplinary programs in molecular biophysics and biomolecular structure and design. I started expanding the notes partly to try to illuminate the stream of new experimental information on photosynthetic antennas and reaction centers, but mostly just for fun. I hope that readers will find the results not only useful, but also as stimulating as I have.

One of my goals has been to write in a way that will be accessible to readers with little prior training in quantum mechanics. But any contemporary discussion of how light interacts with molecules must begin with quantum mechanics, just as experimental observations on blackbody radiation, interference, and the photoelectric effect form the springboard for almost any introduction to quantum mechanics. To make the reasoning as transparent as possible, I have tried to adopt a consistent theoretical approach, minimize jargon, and explain any terms or mathematical methods that might be unfamiliar. I have provided numerous figures to relate spectroscopic properties to molecular structure, dynamics, and electronic and vibrational wavefunctions. I also describe classical pictures in many cases and indicate where these either have continued to be useful or have been supplanted by quantum mechanical treatments. Readers with experience in quantum mechanics should be able to skip quickly through many of the explanations, but will find that the discussion of topics such as density matrices and wavepackets often progresses well beyond the level of a typical 1-year course in quantum mechanics. I have tried to take each topic far enough to provide a solid steppingstone to current theoretical and experimental work in the area.

Although much of the book focuses on physical theory, I have emphasized aspects of optical spectroscopy that are especially pertinent to molecular biophysics, and I have drawn most of the examples from this area. The book therefore covers topics that receive little attention in most general books on molecular spectroscopy, including exciton interactions, resonance energy transfer, single-molecule spectroscopy, high-resolution fluorescence microscopy, femtosecond pump-probe spectroscopy, and photon echoes. It says less than is customary about atomic spectroscopy and about rotational and vibrational spectroscopy of small molecules. These choices reflect my personal interests and the realization that I had to stop somewhere, and I can only apologize to readers whose selections would have been different. I apologize also for using work from my own laboratory in many of the illustrations when other excellent illustrations of

the same points are available in the literature. This was just a matter of convenience.

I could not have written this book without the patient encouragement of my wife Polly. I also have enjoyed many thought-provoking discussions with Arie Warshel, Nagarajan, Martin Gouterman, and numerous other colleagues and students, particularly including Rhett Alden, Edouard Alphonandéry, Hiro Arata, Donner Babcock, Mike Becker, Bob Blankenship, Steve Boxer, Jacques Breton, Jim Callis, Patrik Callis, Rod Clayton, Richard Cogdell, Tom Ebrey, Tom Engel, Graham Fleming, Eric Heller, Dewey Holten, Ethan Johnson, Amanda Jonsson, Chris Kirmaier, David Klug, Bob Knox, Rich Mathies, Eric Merkle, Don Mendenhall, Tom Moore, Jim Norris, Oleg Prezhdo, Phil Reid, Bruce Robinson, Karen Rutherford, Ken Sauer, Dustin Schaefer, Craig Schenck, Peter Schellenberg, Avigdor Scherz, Mickey Schurr, Gerry Small, Rienk van Grondelle, Maurice Windsor, and Neal Woodbury. Patrik Callis kindly provided the atomic coefficients used in Chaps. 4 and 5 for the molecular orbitals of 3-methylindole. Any errors, however, are entirely mine. I will appreciate receiving any corrections or suggestions for improvements.

Seattle, October 2006

William W. Parson

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1 Introduction

1.1

Overview

Because of their extraordinary sensitivity and speed, optical spectroscopic techniques are well suited for addressing a broad range of questions in molecular and cellular biophysics. Photomultipliers sensitive enough to detect a single photon make it possible to measure the fluorescence from individual molecules, and lasers providing light pulses with widths of less than 10^{-14} s can be used to probe molecular behavior on the time scale of nuclear motions. Spectroscopic properties such as absorbance, fluorescence, and linear and circular dichroism can report on the identities, concentrations, energies, conformations, or dynamics of molecules and can be sensitive to small changes in molecular structure or surroundings. Resonance energy transfer provides a way to probe intermolecular distances. Because they usually are not destructive, spectrophotometric techniques can be used with samples that must be recovered after an experiment. They also can provide analytical methods that avoid the need for radioisotopes or hazardous reagents. When combined with genetic engineering and microscopy, they provide windows to the locations, dynamics, and turnover of particular molecules in living cells.

In addition to describing applications of optical spectroscopy in biophysics and biochemistry, this book is about light and how light interacts with matter. These are topics that have puzzled and astonished people for thousands of years, and continue to do so today. To understand how molecules respond to light we first must inquire into why molecules exist in well-defined states and how they change from one state to another. Thinking about these questions underwent a series of revolutions with the development of quantum mechanics, and today quantum mechanics forms the scaffold for almost any investigation of molecular properties. Although most of the molecules that interest biophysicists are far too large and complex to be treated exactly by quantum mechanical techniques, their properties often can be rationalized by quantum mechanical principles that have been refined on simpler systems. We will discuss these principles in Chap. 2. For now, the most salient points are just that a molecule can exist in a variety of states depending on how its electrons are distributed among a set of molecular orbitals, and that each of these states is associated with a definite energy. For a molecule with $2n$ electrons, the electronic state with the lowest total energy usually is obtained when there are two electrons with antiparallel spins in each of the n lowest orbitals and all the higher orbitals are empty. This is the *ground state*. In the absence of

external perturbations, a molecule placed in the ground state will remain there indefinitely.

Chapter 3 will discuss the nature of light, beginning with a classical description of an oscillating electromagnetic field. Exposing a molecule to such a field causes the potential energies of the electrons to fluctuate with time, so that the original molecular orbitals no longer limit the possibilities. The result of this can be that an electron moves from one of the occupied molecular orbitals to an unoccupied orbital with a higher energy. Two main requirements must be met in order for such a transition to occur. First, the electromagnetic field must oscillate at the right frequency. The required frequency (ν) is

$$\nu = \Delta E/h, \quad (1.1)$$

where ΔE is the difference between the energies of the ground and excited states and h is Planck's constant (6.63×10^{-34} J s, 4.12×10^{-15} eV s, or 3.34×10^{-11} cm⁻¹ s). This expression is in accord with our experience that a given type of molecule, or a molecule in a particular environment, absorbs light of some colors and not of others. In Chap. 4 we will see that the frequency rule emerges straightforwardly from the classical electromagnetic theory of light, as long as we treat the absorbing molecule quantum mechanically. It is not necessary at this point to use a quantum mechanical picture of light.

The second requirement is perhaps less familiar than the first, and has to do with the shapes of the two molecular orbitals and the disposition of the orbitals in space relative to the polarization of the oscillating electrical field. The two orbitals must have different geometrical symmetries and must be oriented in an appropriate way with respect to the field. This requirement rationalizes the observation that absorption bands of various molecules vary widely in strength. It also explains why the absorbance of an anisotropic sample depends on the polarization of the light beam.

The molecular property that determines both the strength of an absorption band and the optimal polarization of the light is a vector called the *transition dipole*, which can be calculated from the molecular orbitals of the ground and the excited state. The square of the magnitude of the transition dipole is termed the *dipole strength*, and is proportional to the strength of absorption. Chapter 4 develops these notions more fully and examines how they arise from the principles of quantum mechanics. This provides the theoretical groundwork for discussing how measurements of the wavelength, strength, or polarization of electronic absorption bands can provide information on molecular structure and dynamics. In Chaps. 10 and 11 we extend the quantum mechanical treatment of absorption to large ensembles of molecules that interact with their surroundings in a variety of ways. Various types of vibrational spectroscopy are discussed in Chaps. 6 and 12.

A molecule that has been excited by light can decay back to the ground state by several possible paths. One possibility is to reemit energy as fluorescence. Although spontaneous fluorescence is not simply the reverse of absorption, it shares the same requirements for energy matching and appropriate orbital symmetry. Again, the

frequency of the emitted radiation is proportional to the energy difference between the excited and ground states and the polarization of the radiation depends the orientation of the excited molecule, although both the orientation and the energy of the excited molecule usually change in the interval between absorption and emission. As we will see in Chap. 7, the same requirements underlie another mechanism by which an excited molecule can decay, the transfer of energy to a neighboring molecule. The relationship between fluorescence and absorption is developed in Chap. 5, where the need for a quantum theory of light finally comes to the front.

1.2

The Beer–Lambert Law

A beam of light passing through a solution of absorbing molecules transfers energy to the molecules as it proceeds, and thus decreases progressively in intensity. The decrease in the intensity, or irradiance (I), over the course of a small volume element is proportional to the irradiance of the light entering the element, the concentration of absorbers (C), and the length of the path through the element (dx):

$$\frac{dI}{dx} = -\epsilon' I C . \quad (1.2)$$

The proportionality constant (ϵ') depends on the wavelength of the light and on the absorber's structure, orientation and environment. Integrating Eq. (1.2) shows that if light with irradiance I_0 is incident on a cell of thickness l , the irradiance of the transmitted light will be

$$I = I_0 \exp(-\epsilon' C l) = I_0 10^{-\epsilon C l} \equiv I_0 10^{-A} . \quad (1.3)$$

Here A is the *absorbance* or *optical density* of the sample ($A = \epsilon C l$) and ϵ is called the *molar extinction coefficient* or *molar absorption coefficient* ($\epsilon = \epsilon' / \ln 10 = \epsilon' / 2.303$). The absorbance is a dimensionless quantity, so if C is given in units of molarity ($1 \text{ M} = 1 \text{ mol l}^{-1}$) and c in cm, ϵ must have dimensions of $\text{M}^{-1} \text{ cm}^{-1}$.

Equations (1.1) and (1.2) are statements of *Beer's law*, or more accurately, the *Beer–Lambert law*. Johann Lambert, a physicist, mathematician, and astronomer born in 1728, observed that the fraction of the light that is transmitted (I/I_0) is independent of I_0 . Wilhelm Beer, a banker and astronomer who lived from 1797 to 1850, noted the exponential dependence on C .

In the classical electromagnetic theory of light, the oscillation frequency (ν) is related to the wavelength (λ), the velocity of light in a vacuum (c), and the refractive index of the medium (n) by the expression

$$\nu = c/n\lambda . \quad (1.4)$$

Light with a single wavelength, or more realistically, with a narrow band of wavelengths, is called *monochromatic*.

The light intensity, or *irradiance* (I), in Eqs. (1.2) and (1.3) represents the flux of radiant energy per unit cross-sectional area of the beam (joules per second per square centimeter or watts per square centimeter). We usually are concerned with the radiation in a particular frequency interval ($\Delta\nu$), so I has units of joules per frequency interval per second per square centimeter. For a light beam with a cross-sectional area of 1 cm^2 , the amplitude of the signal that might be recorded by a photomultiplier or other detector is proportional to $I(\nu)\Delta\nu$. In the quantum theory of light that we will discuss briefly in Sect. 1.6 and at greater depth in Chap. 3, intensities often are expressed in terms of the flux of photons rather than energy (photons per frequency interval per second per square centimeter). A beam with an irradiance of 1 W cm^{-2} has a photon flux of $5.05 \times (\lambda/\text{nm}) \times 10^{15}$ photons cm^{-2} .

The dependence of the absorbance on the frequency of light can be displayed by plotting A or ϵ as a function of the frequency (ν), the wavelength (λ), or the *wavenumber* ($\bar{\nu}$). The wavenumber is simply the reciprocal of the wavelength in a vacuum: $\bar{\nu} = 1/\lambda = \nu/c$, and has units of cm^{-1} . Sometimes the percentage of the incident light that is absorbed or transmitted is plotted. The percentage absorbed is $100 \times (I_0 - I)/I_0 = 100 \times (1 - 10^{-A})$, which is proportional to A if $A \ll 1$.

1.3

Regions of the Electromagnetic Spectrum

The regions of the electromagnetic spectrum that will be most pertinent to our discussion involve wavelengths between 10^{-9} and 10^{-2} cm. Visible light fills only the small part of this range between 3×10^{-5} and 8×10^{-5} cm (Fig. 1.1). Transitions of bonding electrons occur mainly in this region and the neighboring UV region; vibrational transitions occur in the IR. Rotational transitions are measurable in the far-IR region in small molecules, but in macromolecules these transitions are too congested to resolve. Radiation in the X-ray region can cause transitions in which $1s$ or other core electrons are excited to atomic $3d$ or $4f$ shells or are dislodged completely from a molecule. These transitions can report on the oxidation and coordination states of metal atoms in metalloproteins.

The inherent sensitivity of absorption measurements in different regions of the electromagnetic spectrum decreases with increasing wavelength because, in the idealized case of a molecule that absorbs and emits radiation at a single frequency, it depends on the difference between the populations of molecules in the ground and excited states. If the two populations are the same, radiation at the resonance frequency will cause upward and downward transitions at the same rate, giving a net absorbance of zero. At thermal equilibrium, the fractional difference in the populations is given by

$$\frac{(N_g - N_e)}{(N_g + N_e)} = \frac{[1 - (S_e/S_g) \exp(-\Delta E/k_B T)]}{[1 + (S_e/S_g) \exp(-\Delta E/k_B T)]} \approx \frac{[1 - \exp(-h\nu/k_B T)]}{[1 + \exp(-h\nu/k_B T)]}, \quad (1.5)$$

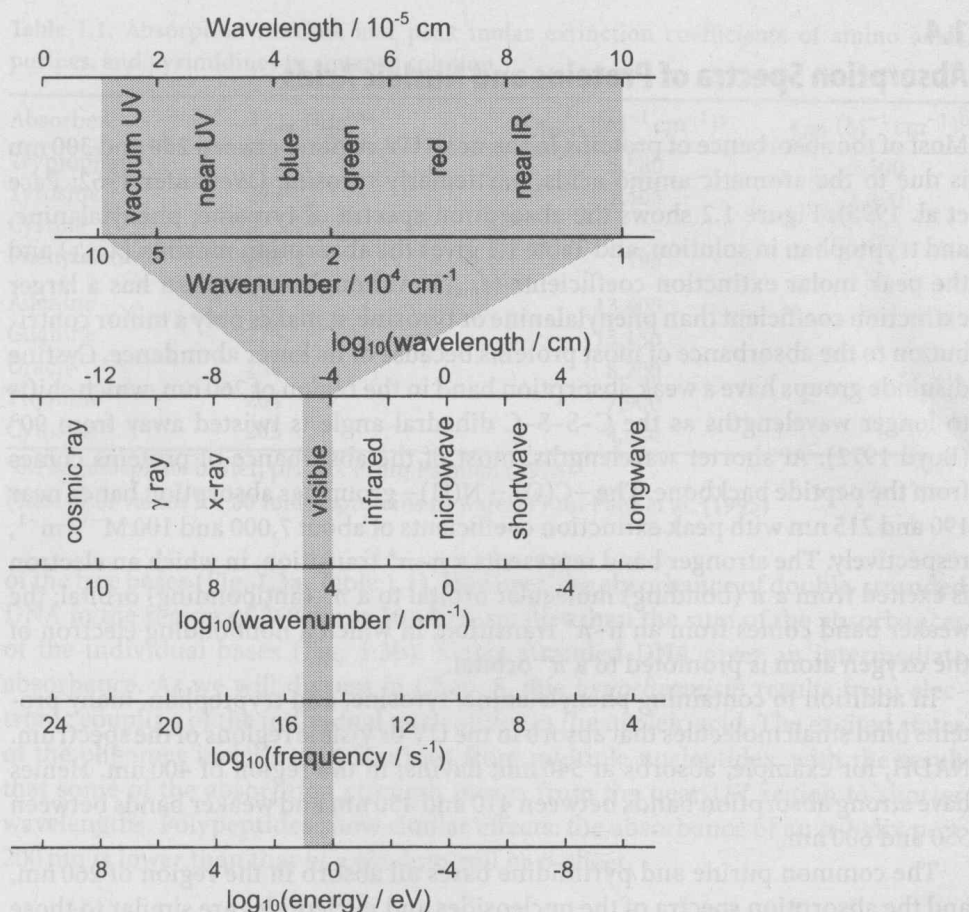


Fig. 1.1. Regions of the electromagnetic spectrum. The *upper spectrum* shows the visible, UV, and near-IR regions on a linear wavelength scale. More extended spectra are shown on logarithmic wavelength, wavenumber, frequency, and energy scales

where S_e/S_g is an entropic (degeneracy) factor (1 for a system with only two states), k_B is the Boltzmann constant ($0.69502 \text{ cm}^{-1} \text{ K}^{-1}$), and T is the temperature. At room temperature ($k_B T \approx 200 \text{ cm}^{-1}$), $(N_g - N_e)/(N_g + N_e)$ is essentially 1 for an electronic transition with $\lambda = 500 \text{ nm}$ [$h\nu = \bar{\nu} = 2 \times 10^4 \text{ cm}^{-1}$, $\exp(-h\nu/k_B T) \approx \exp(-100) \approx 10^{-43}$], compared with only 1 part in 10^4 for a proton magnetic transition in a 600-MHz spectrometer [$\lambda = 50 \text{ cm}$, $\bar{\nu} = 0.02 \text{ cm}^{-1}$, $\exp(-h\nu/k_B T) \approx e^{-0.00010} \approx 0.99990$]. The greater specificity of NMR of course often compensates for the lower sensitivity.

1.4

Absorption Spectra of Proteins and Nucleic Acids

Most of the absorbance of proteins in the near-UV region between 250 and 300 nm is due to the aromatic amino acids, particularly tyrosine (Wetlaufer 1962; Pace et al. 1995). Figure 1.2 shows the absorption spectra of tyrosine, phenylalanine, and tryptophan in solution, and Table 1.1 gives the absorption maxima (λ_{\max}) and the peak molar extinction coefficients (ϵ_{\max}). Although tryptophan has a larger extinction coefficient than phenylalanine or tyrosine, it makes only a minor contribution to the absorbance of most proteins because of its lower abundance. Cystine disulfide groups have a weak absorption band in the region of 260 nm, which shifts to longer wavelengths as the C-S-S-C dihedral angle is twisted away from 90° (Boyd 1972). At shorter wavelengths, most of the absorbance of proteins comes from the peptide backbone. The $-\text{C}(\text{O})-\text{N}(\text{H})-$ group has absorption bands near 190 and 215 nm with peak extinction coefficients of about 7,000 and $100 \text{ M}^{-1} \text{ cm}^{-1}$, respectively. The stronger band represents a $\pi-\pi^*$ transition, in which an electron is excited from a π (bonding) molecular orbital to a π^* (antibonding) orbital; the weaker band comes from an $n-\pi^*$ transition, in which a nonbonding electron of the oxygen atom is promoted to a π^* orbital.

In addition to containing phenylalanine, tyrosine, and tryptophan, many proteins bind small molecules that absorb in the UV or visible regions of the spectrum. NADH, for example, absorbs at 340 nm; flavins, in the region of 400 nm. Hemes have strong absorption bands between 410 and 450 nm and weaker bands between 550 and 600 nm.

The common purine and pyrimidine bases all absorb in the region of 260 nm, and the absorption spectra of the nucleosides and nucleotides are similar to those

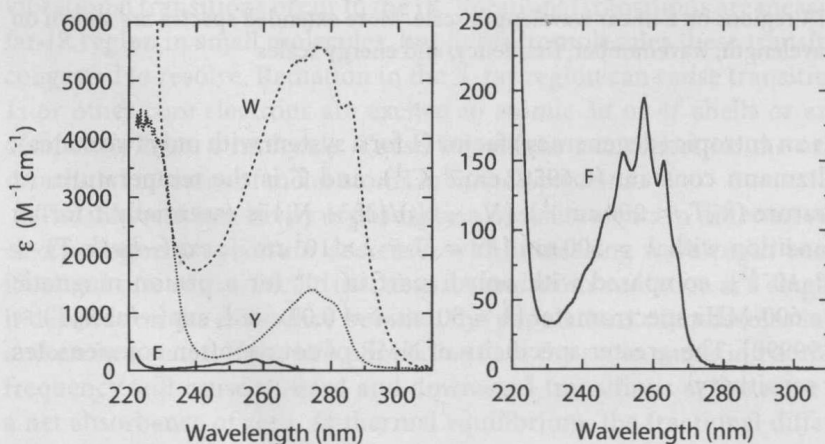


Fig. 1.2. Absorption spectra of phenylalanine (F), tyrosine (Y), and tryptophan (W) in 0.1 M phosphate buffer, pH 7. The spectrum of phenylalanine is shown on an expanded scale on the right. (The data are from a library of spectra measured by Lindsey and coworkers (Du et al. 1998; Dixon et al. 2005))

Table 1.1. Absorption maxima and peak molar extinction coefficients of amino acids, purines, and pyrimidines in aqueous solution

Absorber	λ_{\max} (nm) ^a	ϵ_{peak} (M ⁻¹ cm ⁻¹) ^a	ϵ_{280} (M ⁻¹ cm ⁻¹) ^b
Tryptophan	278	5,580	5,500
Tyrosine	274	1,405	1,490
Cystine	—	—	125
Phenylalanine	258	195	
Adenine	261	13,400	
Guanine	273	13,150	
Uracil	258	8,200	
Thymine	264	7,900	
Cytosine	265	4,480	

^a0.1 M phosphate buffer, pH 7.0. From Fasman (1976)

^bBest fit of values for 80 folded proteins in water. From Pace et al. (1995)

of the free bases (Fig. 1.3a, Table 1.1). However, the absorbance of double-stranded DNA in the region of 260 nm is 30–40% smaller than the sum of the absorbances of the individual bases (Fig. 1.3b). Single-stranded DNA gives an intermediate absorbance. As we will discuss in Chap. 8, this *hypochromism* results from electronic coupling of the individual nucleotides in the nucleic acid. The excited states of the oligomer include contributions from multiple nucleotides, with the result that some of the absorption strength moves from the near-UV region to shorter wavelengths. Polypeptides show similar effects: the absorbance of an α -helix near 200 nm is lower than that of a random coil or β -sheet.

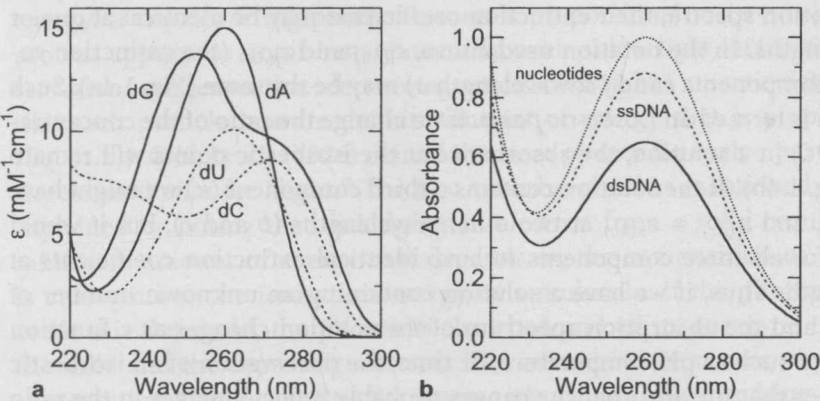


Fig. 1.3. a Absorption spectra of 2'-deoxyadenosine (dA), 2'-deoxyguanosine (dG), 2'-deoxyuridine (dU) and 2'-deoxycytidine (dC) at pH 7.1 b Absorption spectra of *Escherichia coli* DNA at 25 °C (double-stranded DNA, *ds*-DNA) and 82 °C (single-stranded DNA, *ss*-DNA), and at 25 °C after enzymatic digestion (*nucleotides*). *E. coli* DNA is double-stranded at 25 °C and single-stranded at 82 °C; enzymatic digestion yields the component nucleotides. (The data in b are from Voet et al. 1963.)

1.5

Absorption Spectra of Mixtures

An important corollary of the Beer–Lambert law (Eq. (1.1)) is that the absorbance of a mixture of noninteracting molecules is just the sum of the absorbances of the individual components. This means that the absorbance change resulting from a change in the concentration of one of the components is independent of the absorbance due to the other components. In principle, we can determine the concentrations of all the components by measuring the absorbance of the solution at a set of wavelengths where the molar extinction coefficients of the components differ. The concentrations (C_i) are obtained by solving the simultaneous equations

$$\begin{aligned}\epsilon_{1(\lambda_1)}C_1 + \epsilon_{2(\lambda_1)}C_2 + \epsilon_{3(\lambda_1)}C_3 + \cdots &= A_{\lambda_1}/l \\ \epsilon_{1(\lambda_2)}C_1 + \epsilon_{2(\lambda_2)}C_2 + \epsilon_{3(\lambda_2)}C_3 + \cdots &= A_{\lambda_2}/l \\ \epsilon_{1(\lambda_3)}C_1 + \epsilon_{2(\lambda_3)}C_2 + \epsilon_{3(\lambda_3)}C_3 + \cdots &= A_{\lambda_3}/l \\ \cdots, &\end{aligned}\tag{1.6}$$

where $\epsilon_{i(\lambda_a)}$ and A_{λ_a} are the molar extinction coefficient of component i and the absorbance of the solution at wavelength λ_a , and l again is the optical path length. (A method for solving such a set of equations is given in Box 8.1.) The concentrations are completely determined when the number of measurement wavelengths is the same as the number of components, as long as the extinction coefficients of the components differ significantly at each wavelength. Measurements at additional wavelengths can be used to increase the reliability of the results. The best way to calculate the concentrations then probably is to use singular-value decomposition (Press et al. 1989).

Although two chemically distinct molecules usually have characteristically different absorption spectra, their extinction coefficients may be identical at one or more wavelengths. In the notation used above, $\epsilon_{i(\lambda_a)}$ and $\epsilon_{j(\lambda_a)}$ (the extinction coefficients of components i and j at wavelength a) may be the same (Fig. 1.4a). Such a wavelength is termed an *isosbestic point*. If we change the ratio of the concentrations C_i and C_j in a solution, the absorbance at the isosbestic points will remain constant (Fig. 1.4b). If the solution contains a third component (k) we might have $\epsilon_{i(\lambda_b)} = \epsilon_{k(\lambda_b)}$ and $\epsilon_{j(\lambda_c)} = \epsilon_{k(\lambda_c)}$ at two other wavelengths (b and c), but it would be unlikely for all three components to have identical extinction coefficients at any wavelength. Thus, if we have a solution containing an unknown number of components and the absorption spectrum of the solution changes as a function of a parameter such as pH, temperature, or time, the observation of an isosbestic point indicates that the absorbance changes probably reflect changes in the ratio of only two components. The reliability of this conclusion increases if there are two isosbestic points.

Protein concentrations often are estimated from the absorbance at 280 nm, where the only amino acids that absorb significantly are tryptophan, tyrosine, and cystine (Table 1.1). The molar extinction coefficient of a protein at this wavelength is given by $\epsilon_{280} \approx 5,500 \times W + 1,490 \times Y + 125 \times CCM^{-1} \text{ cm}^{-1}$, where W , Y , and CC