

Spectroscopic Methods in Biochemistry — Principles and Applications

(Spektroskopische Methoden in der Biochemie — Grundlagen und
Anwendungen.)

© Jörg H. Kleinschmidt
WS 2000/2001

redistribution, duplication and modification of this manuscript are allowed only with my explicit permission

Weitergabe, Vervielfältigung oder Änderung dieses Textes nur mit meiner ausdrücklichen Erlaubnis.

This text is intended for students of basic sciences who are interested in the application and principles of spectroscopic techniques in biochemical and biophysical research. It is intended as an introduction into commonly used spectroscopic methods, their very basic principles and their use to investigate structure, function and dynamics of biological systems. In its present form, this text should be considered as an incomplete draft that will be updated on a regular basis. As a draft, this text may still contain errors or paragraphs that need improvements in clarity. The author therefore welcomes corrections and suggestions at any time.

1. Introduction

Absorption and Emission of electromagnetic radiation by matter are very important processes not only for the study of biological systems, but also for the function of life as we know it: Without the interaction of light and chromophores, there would be no visual perception (we would not see) and plants would not be able to perform photosynthesis to produce sugars and other carbohydrates. These processes involve the interaction of matter with visible electromagnetic radiation. However, in the study of biological structure, function and dynamics, the interaction between radiation and matter is not limited to the visible region of the electromagnetic spectrum. In biological spectroscopy, radiation of a wavelength between kilometers and picometers has been used successfully. Table 1 gives an overview over spectroscopic techniques that will be discussed in this lecture and the corresponding parameters of the electromagnetic radiation.

1.1. Examples of Radiation Sources

There are many sources for radiation. For example, visible radiation (“Light”) is generated by chemical reactions and energy transformations in the flame of burning substances.

On the sun, our daylight is generated by nuclear fusion of hydrogen to helium and a broad range of radiation is emitted that extends beyond the visible region. Fortunately, the atmosphere absorbs most of the energetic light such as the ultraviolet (UV) radiation that is dangerous for the life on our planet.

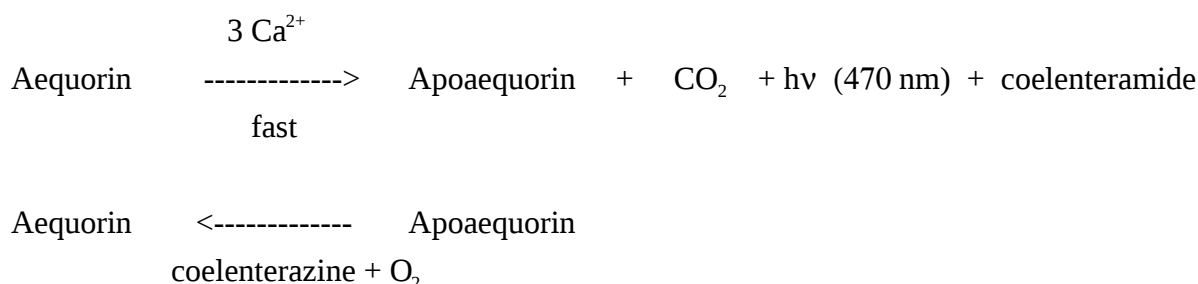
Electromagnetic radiation is generated by electrical discharge (for example in form of lightning in a thunderstorm) or in a xenon lamp in which an bright lightbow is generated in a lightbulb that is filled with xenon-gas.

Radiation is also generated by electrical circuitry for example radio, television and communication frequencies (ranging from kHz to GHz. Radio frequencies are very important for the study of structure and function in biology and are used in NMR spectroscopy (typically between 60 to 900 MHz). The basic electronic parts to design an oscillator for the generation of radio waves are the capacitor and the inductor.

Certain Animals are also able to generate electromagnetic radiation

Examples are the firefly and the jellyfish. The luminescent jellyfish (*aequorea victoria*) is capable of producing flashes of blue light (470 nm). These flashes are produced by an intracellular increase in the Ca^{2+} concentration that causes the transition of aequorin to apoaequorin and CO_2 and light. The blue light is then transduced to green (502 nm) by the

famous green fluorescent protein.



The process is catalyzed by an enzyme called luciferase. A similar luciferase catalyzes the conversion of the protein luciferin to oxyluciferin in the firefly.

Green fluorescent protein, GFP, is a spontaneously fluorescent protein isolated from coelenterates, such as the Pacific jellyfish, *Aequoria victoria*. Its role is to transduce, by energy transfer, the blue bioluminescence of aequorin into green fluorescent light. The molecular cloning of GFP cDNA and the demonstration by Chalfie that GFP can be expressed as a functional transgene, have opened exciting new avenues of investigation in cell, developmental and molecular biology. Fluorescent GFP has been expressed in bacteria, yeast, slime mold, plants, drosophila, zebrafish, and in mammalian cells. GFP can function as a protein tag, as it tolerates N- and C-terminal fusion to a broad variety of proteins many of which have been shown to retain native function. When expressed in mammalian cells fluorescence from wild type GFP is typically distributed throughout the cytoplasm and nucleus, but excluded from the nucleolus and vesicular organelles. However, highly specific intracellular localization including the nucleus, mitochondria, secretory pathway, plasma membrane and cytoskeleton can be achieved via fusions both to whole proteins and individual targeting sequences. The enormous flexibility as a noninvasive marker in living cells allows for numerous other applications such as a cell lineage tracer, reporter of gene expression and as a potential measure of protein-protein interactions.

1.2. Characterization of radiation.

All radiation is characterized by a wavelength, an amplitude and velocity of propagation, Energy (E), frequency (ν), wavelength (λ), and wavenumber ($\tilde{\nu}$) are related by the following equations (see also Figure 1.1):

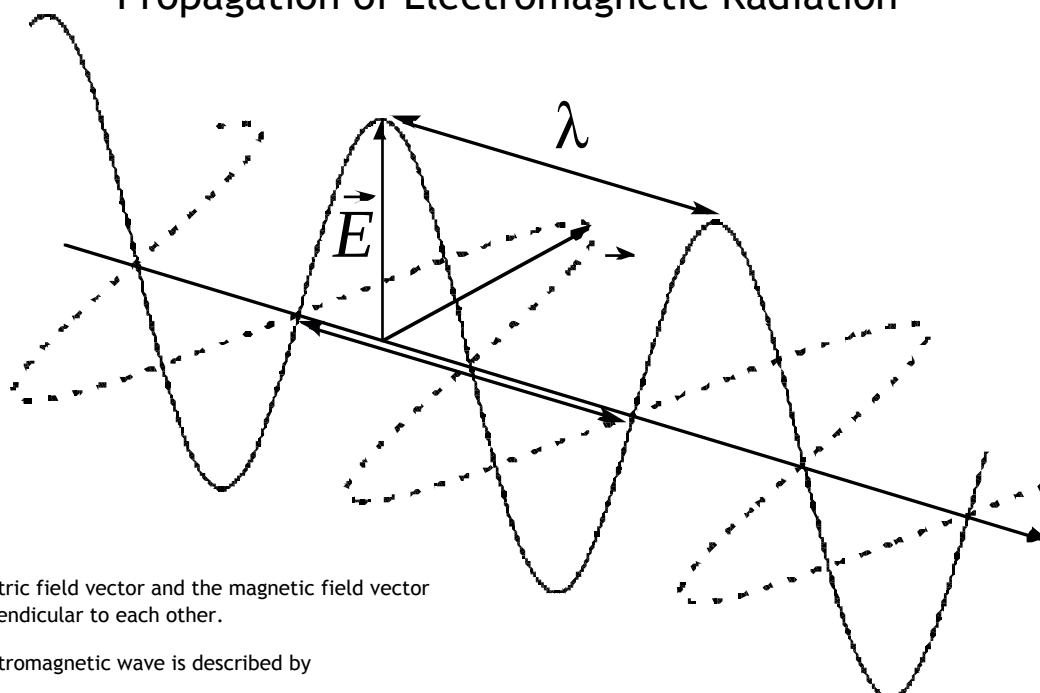
$$E = h \cdot \nu, \quad h = 6.626 \times 10^{-34} \text{ Js, Planck's Constant}$$

$$c = \nu \cdot \lambda, \quad c = 2.998 \times 10^8 \text{ ms}^{-1}, \text{ Velocity of Radiation in Vacuum}$$

$$\tilde{\nu} = \frac{1}{100 \times \lambda}$$

Note that the wavenumber is usually given in reciprocal centimeters.

Propagation of Electromagnetic Radiation



The electric field vector and the magnetic field vector are perpendicular to each other.

The electromagnetic wave is described by

$$E = E_0 \sin(2\pi \nu t) \text{ and } B = B_0 \sin(2\pi \nu t),$$

respectively.

ν : Frequency: the number of wavelengths the radiation travels in 1s.

t : time

E_0, B_0 : amplitudes

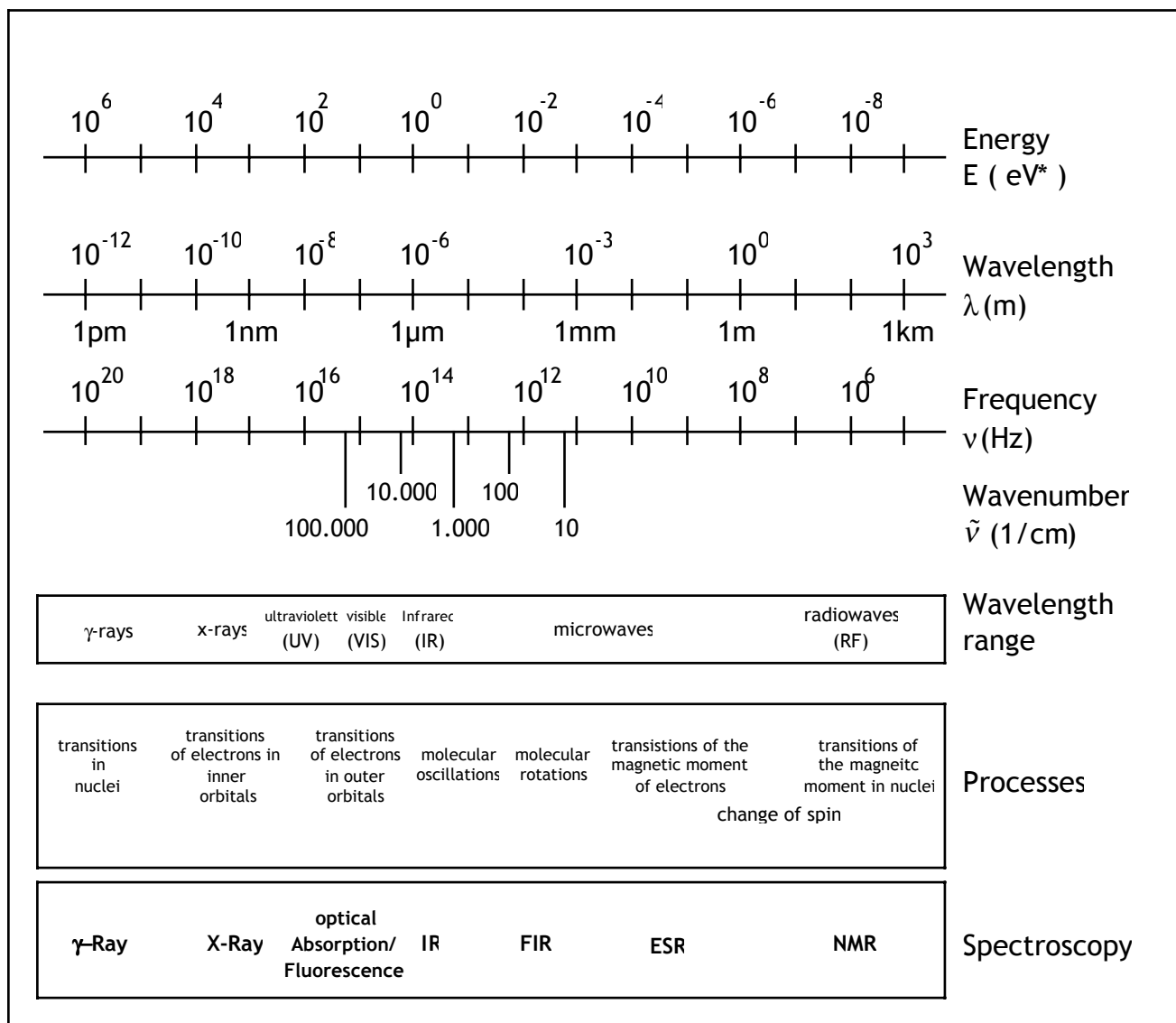
Figure 1.1 Propagation of electromagnetic radiation

1.3. Qualitative description of the interaction of electromagnetic radiation and matter.

The interaction of radiation and matter is characterized by an alteration in the distribution of molecular charges or spins that have electrical and magnetic properties. In spectroscopy, radiation is sent through a sample either continuously or in form of a pulse. Depending on the kind of radiation the molecule may react to the perturbation. Explanations must be found, why only a certain kind of radiation is absorbed, how fast the molecule reacts to the perturbation, and how matter alters the properties of the radiation.

Spectroscopy

Interaction of Electromagnetic Radiation and Matter



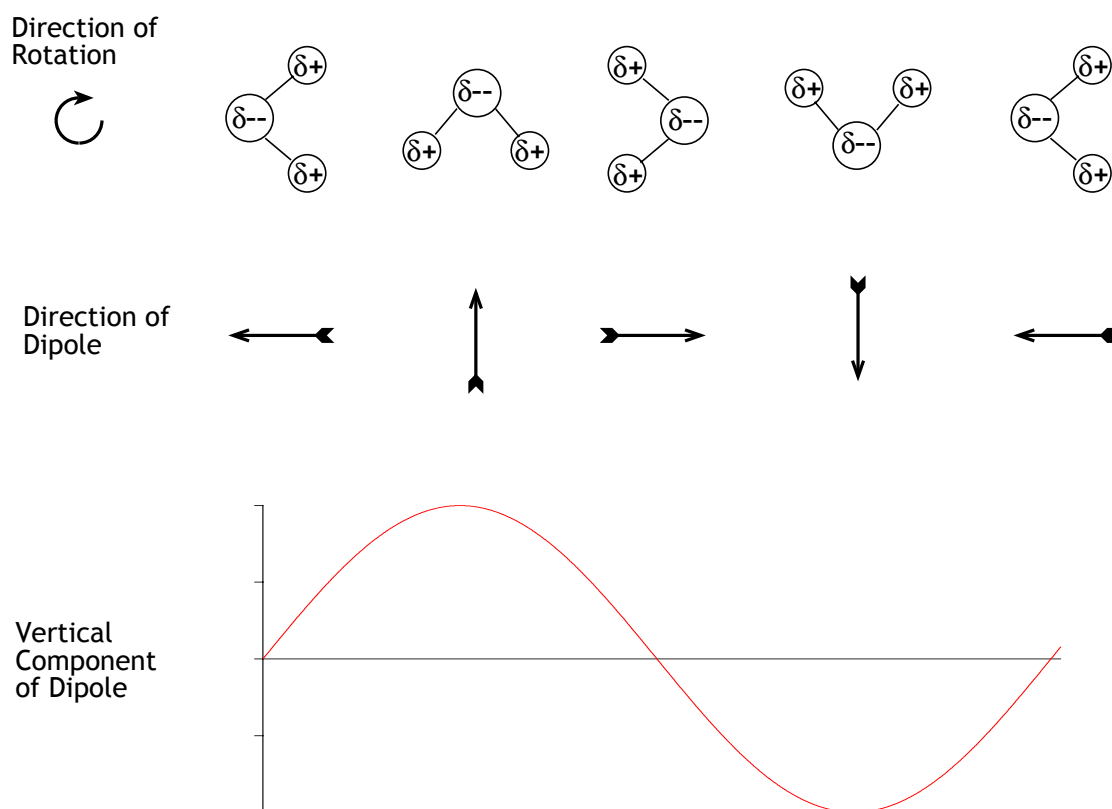
*) $1 \text{ eV} = 1.602 \times 10^{-19} \text{ J} = 3.83 \times 10^{-20} \text{ cal}$

Figure 1.2 Electromagnetic radiation and spectroscopic techniques

1.4. Regions of the spectrum

A. Radio frequency (rf) region: ranges from MHz (km -waves) to about 50 GHz (cm waves). These frequencies are used in nuclear magnetic resonance spectroscopy (NMR, MHz) and in electron paramagnetic resonance spectroscopy (EPR, also called electron spin resonance spectroscopy, ESR, GHz region). The energy change involved is that arising from the reversal of spin of a nucleus or electron and is of the order of 0.001 to 20 J/mol. The spin is associated with a tiny magnetic dipole and the spin-reversal a result of the interaction of this dipole with the magnetic field of the electromagnetic radiation at the appropriate frequency.

B. Microwave region: 30 GHz to 3×10^{12} Hz (1 cm to 100 μm wavelength). Rotational spectroscopy. Separations between the rotational levels of molecules are of the order 100 J/mol. In Microwave spectroscopy, molecules that can interact with electromagnetic radiation must be polar, i.e. one part of the molecule must carry a permanent positive partial charge and another part a permanent positive partial charge. An example is the water molecule, in which the oxygen carries a negative partial charge and the hydrogens carry a positive partial charge. Therefore the water molecule is a dipole. The rotation of this dipole results in aperiodic change of the location of the partial plus and minus charges of the water molecule and the component dipole moment therefore oscillates regularly in a given direction. Molecules that do not have a dipolar moment, for example O_2 , Cl_2 , H_2 , are not microwave active.



The rotation of the water molecule and the corresponding change in the dipole moment along a particular direction

Figure 1.3 Rotational spectra require a permanent dipole moment of a molecule. Shown is the rotation of the water molecule and the corresponding change in the dipole moment along a particular direction

C. Infrared region: 3×10^{12} to 3×10^{14} Hz (100 to 1 μm wavelength). Vibrational spectroscopy. A very useful region for chemists and biochemists. Separations between energy levels are of some 10 kJ/mol. In infrared spectroscopy, the vibration of a molecule must result in a change of the charge distribution in a molecule (i.e. a change in the dipolar momentum).

The carbon dioxide, CO_2 , molecule shall serve as an example.

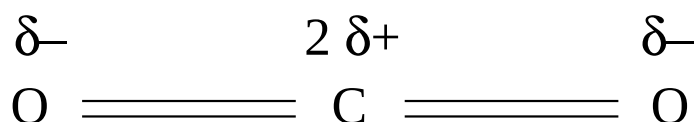
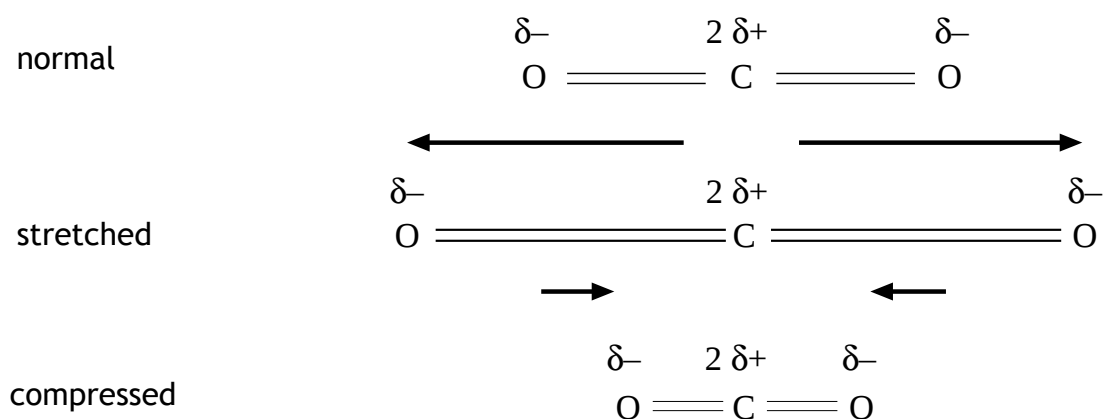


Figure 1.4 Partial charges within the CO_2 molecule.

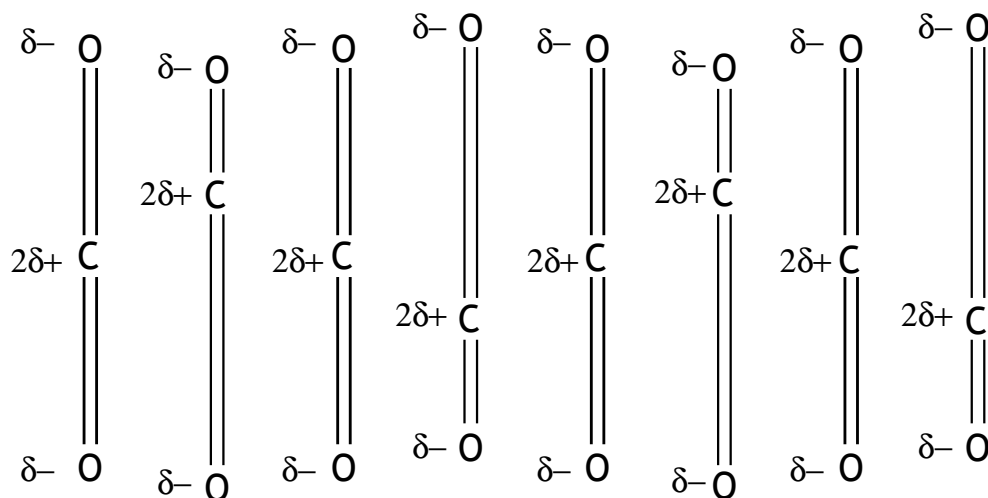
Three atoms are arranged linearly with a small positive charge on the carbon and small negative charges on the oxygen atoms. In a symmetric “stretch vibration” the molecule is alternately stretched and compressed, both C—O bonds are changing simultaneously. During this vibration the dipole moment remains zero throughout the whole motion and this particular vibration is infrared inactive.



Symmetric stretching vibration of the carbon dioxide molecule. No change in the dipolar moment.

Figure 1.5 The symmetric stretching vibration of the carbon dioxide molecule does not produce a change in the dipole moment of the molecule.

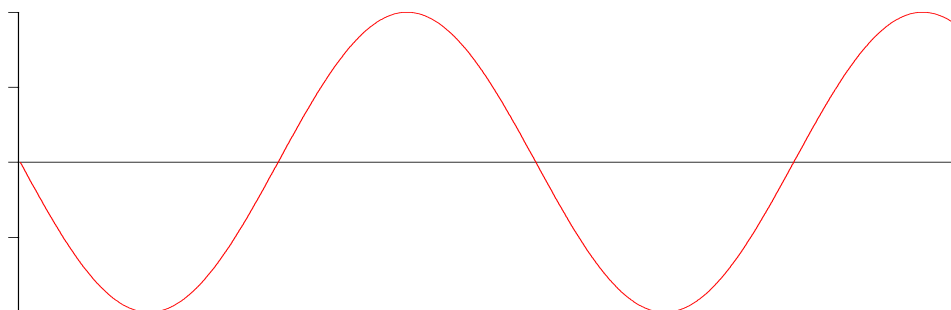
Asymmetric stretching vibration



Dipole Moment



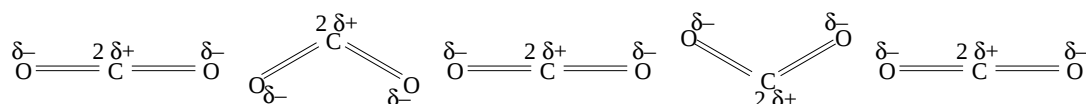
Change of the dipol moment in the course of the asymmetric stretching vibration



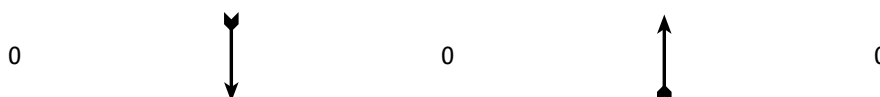
Asymmetric stretching vibration of the carbon dioxide molecule. The dipolar moment depends on the asymmetry and oscillates in the direction of the linear molecule.

Figure 1.6 The asymmetric stretching vibration is accompanied by a change in the dipole moment of the carbon dioxide molecule. Therefore the vibration can be excited by electromagnetic radiation.

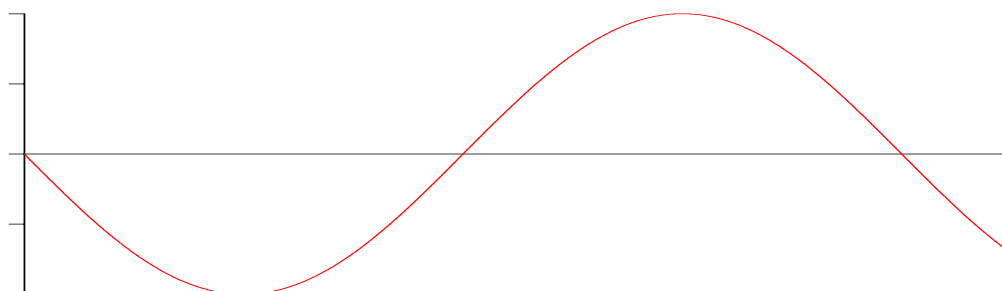
Bending vibration



Dipole Moment



Change of the dipole moment in the course of the asymmetric stretching vibration



Bending vibration of the carbon dioxide molecule and oscillation of the dipole moment.

Figure 1.7. The bending vibration of carbon dioxide and concurrent oscillation of the dipole moment of the molecule.

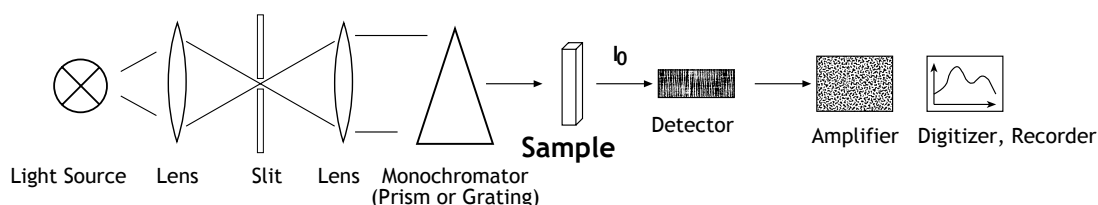
D. Visible and Ultraviolet regions: 3×10^{14} to 3×10^{16} Hz (1 μm to 10 nm). Electronic spectroscopy. The separations between energy levels of valence electrons are some 100 kJ/mol. The excitation of a valence electron results in a charge redistribution and therefore in a change of the dipole moment.

E. X-ray region: 3×10^{16} to 3×10^{18} Hz (10 nm to 100 pm). Spectroscopic transitions that involve the inner electrons of an atom or molecule, and require radiation energies on the order of 10 000 kJ/mol. (German: Röntgen spectroscopy.)

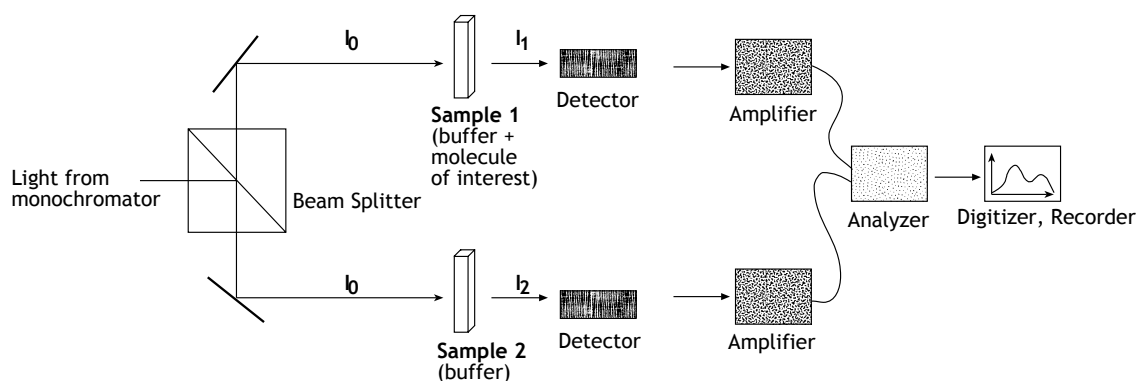
F. γ -ray region: 3×10^{18} to 3×10^{20} Hz (100 pm to 1 pm). Energy transitions involving nuclear particles, about 10^9 to 10^{11} J/mol. Mössbauer spectroscopy.

1.5. Basic configuration of an absorption spectrometer.

A typical instrumental setup for absorption spectroscopy is given below (Figure 1.8). Examples of light sources in absorption spectrometers for ultraviolet or visible light are tungsten or xenon lamps. Infrared light is generated for example by heating silicon carbide. The light generated by such a lamp is composed of radiation of many different frequencies that represents the spectrum of the lamp. To measure the absorption of the sample at a defined wavelength, the light is first focussed by lens on to a narrow slit, then made into a parallel beam by a second lens and subsequently passed through a monochromator, typically a prism or a grating (German: Gitter) that diffracts light depending on its frequency. The monochromatic radiation is then passed through the sample and its intensity is then recorded by the detector, amplified and finally digitized or recorded. By adjustment of the monochromator, the absorbance of the sample can be determined as a function of the wavelength of the radiation: an absorbance spectrum specific for the sample is recorded. The material that is used for lenses, gratings and prisms also depends on the wavelength of the radiation. For example, glass cannot be used in IR instrumentation because it absorbs infrared light.



a Recording light absorption



b Recording difference spectra

Schematic Diagram of a simple absorption spectrometer

Figure 1.8 Block diagram of a typical UV spectrophotometer.

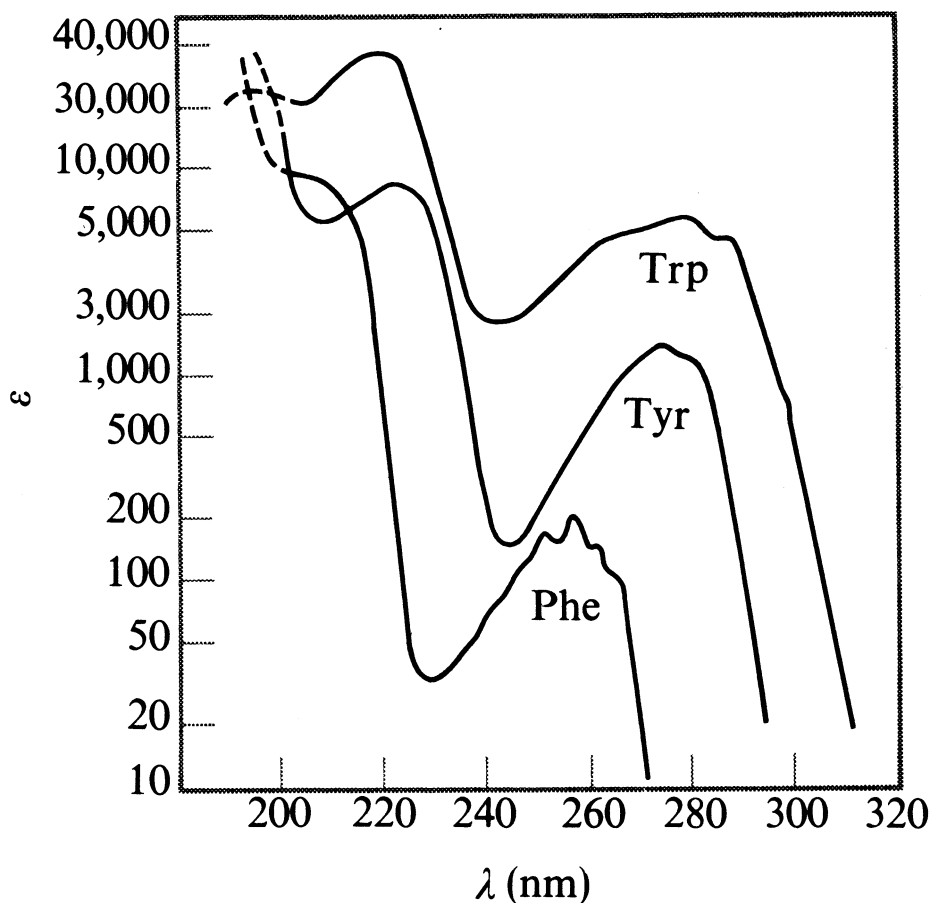


Figure 1.9 UV-absorption spectra of aromatic amino acids

1.6 The Bouguer-Beer Lambert Law. Estimation of concentration.

If radiation of a selected frequency passes through a sample of absorbing molecules M that have the concentration c_M (in mol/l) the absorbance can be calculated. We imagine that the sample is sliced into n layers of a very small thickness dx . The decrease in the absorption dI is proportional to dx and to the concentration c_M . Also when more radiation is sent to the sample more radiation will be absorbed:

$$dI = -\alpha \cdot c_M \cdot I \cdot dx$$

or slightly rearranged

$$\frac{dI}{I} = -\alpha \cdot c_M \cdot dx$$

In this equation, α is a proportionality factor. We can now sum up all the successive changes in the light intensity dI to the total intensity change that is observed over all layers dx :

$$\begin{aligned}\int_{I_0}^I \frac{dI}{I} &= \int_0^l -\alpha \cdot c_M \cdot dx \\ \Rightarrow \ln\left(\frac{I}{I_0}\right) &= -\alpha \cdot c_M \cdot l\end{aligned}$$

or solved for the intensity of the radiation that is observed after passage through the sample:

$$I = I_0 \exp(-\alpha \cdot c_M \cdot l)$$

It is common to express the last equation with base 10 instead of base e (remember

$e^{a \cdot x} = 10^{\log(e^{ax})} = 10^{ax \cdot \log(e)} = 10^{a \cdot \log(e) \cdot x}$, Therefore $\varepsilon = \alpha \cdot \log(e)$):

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

The factor ε is called the molar absorption coefficient (formerly called the ‘extinction coefficient’). The molar absorption coefficient depends on the frequency of the incident radiation and is greatest, when the absorption is most intense. It is therefore sample specific.

The ratio

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

is called the Transmittance T of the sample at a given frequency of the light. The quantity

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

is called the absorbance of the sample at a given frequency. In some older literature, the absorbance A is sometimes called extinction (E) or optical density (OD).