

INTRODUCTION TO SPECTROSCOPY

Literature

- [A] P Atkins, J De Paula: Physical Chemistry, Oxford University Press, 2006
- [C] RMJ Cotterill: Biophysics. An Introduction, chapter 3, John Wiley & Sons, Chichester, 2002
- [CS] CR Cantor, PR Schimmel: Biophysical Chemistry, part II, W H Freeman, NY, 1980 (highly recommended)
- [CD] ID Campbell, RA Dwek, Biological Spectroscopy, 1984 (highly recommended)
- [CDW] N B Colthup, L H Daly, S E Wiberley, *Introduction to infrared and Raman spectroscopy*, 2. Auflage (1975), Academic Press, New York
- [H] JM Hollas: Modern Spectroscopy, John Wiley, 1992
- [HL] W Hoppe, W Lohmann, H Markl, H Ziegler (eds.): *Biophysics*, Springer Verlag, Berlin, 1983
- [HS] FR Hallett, PA Speight, RH Stinson: *Introductory Biophysics*, Methuen, Toronto, 1977
- [Ja] TL James: *Fundamentals of NMR*, Biophysics Textbook Online, 1998
- [M] AG Marshall: Biophysical Chemistry, John Wiley & Sons, New York, 1978

Videos

<https://www.youtube.com/watch?v=dNUkHfBpvac&list=PLm9edRZ1r8wWSE3BruFy06q3ckvjzITfn>

How to read my handouts

Some of my handouts contain supplementary information. These sections are indicated by small print. They represent additional information for those who are interested, but are not required for the examination.

Essential knowledge

Essential knowledge question for Introduction to spectroscopy:

- Restate Fermi's golden rule, explain all the terms and state the significance of the rule. (2 p)
- Define absorbance, or in other words: state how absorbance depends on the measured light intensities. (1 p)
- Write down the Beer-Lambert law and explain all the terms. (1 p)

d) Restate the Boltzmann distribution and explain all the terms and their relevance for the occupancy of the excited state. (2 p)

Examples for general knowledge

1. Define spectroscopy
2. List spectroscopic techniques according to the energy of the transitions observed from low energy transitions to high energy transitions.
3. Name and describe processes that can take place when light interacts with molecules.
4. Explain homogeneous and inhomogeneous line broadening.
5. Which functions describe homogeneous and inhomogeneous line broadening?
6. Give examples for homogeneous and inhomogeneous line broadening
7. Which functions describe homogeneous and inhomogeneous line broadening (no need to learn the equations)?
8. Qualitative comparison of Lorentz and Gauss functions.
9. Restate that $RT = 2.5 \text{ kJ/mol}$ at room temperature.
10. Distinguish between stimulated and spontaneous emission.
11. Compare spectroscopic methods.
12. Very briefly describe the structure and function of bacteriorhodopsin.
13. Appreciate the usefulness of visible spectroscopy for studying bacteriorhodopsin.

Examples for functioning knowledge

1. Apply the Boltzmann distribution to explain spectroscopic properties.
2. Apply the Beer-Lambert law.
3. Predict how the line width of a transition is affected by a given process or change in environment.

What is spectroscopy?

Seeing is spectroscopy: we perceive the world via the interaction of visible light with the light receptors in our eyes. The light is emitted from the sun or from other light sources. It is then reflected from (or transmitted through) the objects in our surroundings. In these processes, the color changes because some of the light is absorbed by the objects. How much and what spectral regions are absorbed depends on the atoms and molecules in these objects. The light not absorbed reaches our eyes. It carries the information of the molecular structure of our surroundings with it. In our eyes its color is analysed by 3 different types of photoreceptors which absorb different light in spectral regions. In this way we perform a spectroscopic experiment every time we look at things. There is a light source, and object that reflects, transmits, scatters and absorbs light and a

wavelength dependent detector in our eyes. An apparatus for spectroscopic studies is called *spectrometer* and a plot of a particular property of matter against wavelength, frequency or energy of radiation is called *spectrum*.

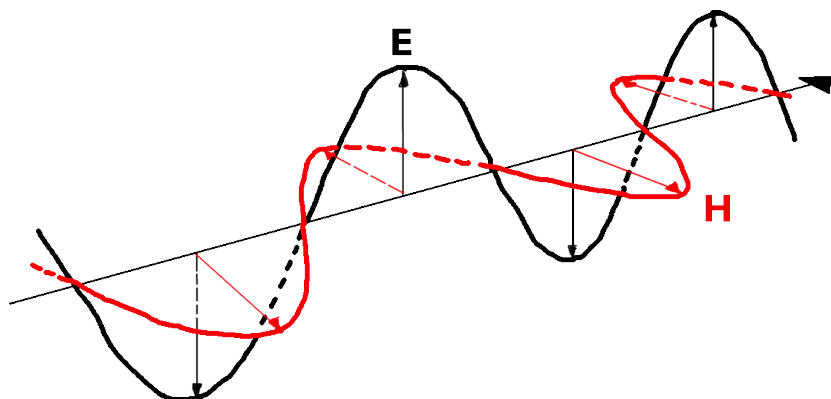
Not only light but also other types of electromagnetic radiation provide powerful information on biological systems. The study of the interaction of electromagnetic radiation with matter is called *spectroscopy* [CD, Wikipedia, Encyclopaedia Britannica, IUPAC Compendium of Chemical Terminology, 2nd ed.]. Because of the wave-particle dualism of matter, spectroscopy includes the related study of the interaction between matter and particles - like electrons and neutrons. With this definition X-ray diffraction, neutron scattering, electron microscopy, and NMR are spectroscopic methods. However, we will not discuss these techniques here, because they are covered in the structural biochemistry course. This course deals instead with the following techniques: UV/vis (ultraviolet/visible) spectroscopy, fluorescence, circular dichroism, Raman spectroscopy, Infrared spectroscopy, and electron spin resonance. Most of these use light in the UV/vis spectral range, infrared spectroscopy uses infrared light and electron spin resonance microwave radiation. In addition to explaining the fundamentals of these techniques, we will also discuss some of their applications to biological systems and biological processes.

The methods that we will discuss are very versatile. In the life sciences they are used to study the structure and dynamics of biomolecules. Apart from the spectroscopy of biological molecules, another impressive example of the power of spectroscopy is at the other extreme of dimensions: the study of space with astronomy. Nearly the only information that we have from outer space reaches us in form of electromagnetic radiation. One example is the recent discovery of water on Mars by the European Mars mission using the infrared spectral region. Other more exotic applications are named in the video. This should not give the impression that spectroscopy is a series of niche techniques. Instead the examples illustrate how wide-spread the use of spectroscopy is.

Outline of the following

In order to extract information from spectroscopic experiments, we need to understand the interaction of radiation with matter. As we will see in more detail below, molecules are perturbed by electromagnetic radiation which makes them change to a state with different energy. Therefore, we need to discuss electromagnetic radiation, energy levels of molecules, the interaction between molecules and radiation and how this leads to transitions between energy levels.

Electromagnetic radiation



*Electric and magnetic components of an electromagnetic wave.
[Cesare Baronio]*

Molecules and their energy levels are one main ingredient of spectroscopy. The other main ingredient is the electromagnetic radiation that induces transitions between different energy levels. Let us therefore briefly recall what electromagnetic radiation is. There are two general ways of describing electromagnetic radiation: as a wave and as a particle. Some aspects of an experiment are best explained by the wave concept, but others by the particle concept. We will use both views to explain the interaction of matter with radiation.

In some experiments and often in the interaction with molecules, electromagnetic radiation behaves particle-like. The particles are called photons. Each photon has a defined energy, which only depends on the frequency (color) of radiation.

$$E = h\nu$$

where h is Planck's constant ($h = 6.63 \times 10^{-34}$ J s). The intensity (brightness) of radiation depends on the number of photons.

If radiation shows its wave face, electromagnetic radiation has two components: an electric field E and a magnetic field B . Both oscillate with the same frequency and are oriented perpendicular to each other and to the direction of propagation at all times. For the phenomena we will describe, it is often sufficient to consider only one of the two components.

Light can be polarized, that is the electric and magnetic field oscillate each in one particular direction. In unpolarized light, the electric and magnetic field oscillate in all directions perpendicular to the direction of propagation.

Frequency ν of a wave and wavelength λ are related by

$$\nu = c/\lambda$$

where c is the velocity of propagation of the wave. For electromagnetic radiation in a vacuum, $c = 3 \times 10^8$ m s⁻¹. Frequency and wavelength are often used to characterize electromagnetic radiation. Another quantity is the wavenumber $\tilde{\nu}$ measured in reciprocal centimeters. The wavenumber is the inverse of the wavelength.

$$\tilde{\nu} = 1/\lambda$$

Wavenumber is mainly used in vibrational spectroscopy. Its advantage is that it conveys the information about the wavelength (just calculate the inverse) and is also proportional to the energy or frequency.

Energy levels

We will now turn to molecules and start discussing their energy levels. A system (molecule) can adopt only certain energy values which are the eigenvalues of its Hamilton operator. Therefore the possible energy values, also called energy levels, are discrete and there are gaps between them. The state of lowest energy is named *ground state*. All states with higher energy are called *excited states*. Sometimes, two states have the same energy, then they are called *degenerate*. This degeneracy can be lifted by a perturbation, i.e. by interaction with an external influence [CD]. An example are the energy levels of the nuclear and electronic spins. In the absence of an external magnetic field, they are degenerate, i.e. the energy does not depend on the spin orientation. However, when an external magnetic field is applied, the degeneracy is lifted and different spin orientations have different energies. One says that the magnetic field splits the spin energy levels.

ENERGY CONTRIBUTIONS

Often one can consider a molecule as being composed of several sub-systems (electron orbitals, electron spin, nuclear vibrations, nuclear spin, etc.) that are quite independent from each other. For example, one can consider the electron orbitals separately from the nuclear spin orientation. This is an approximation of the real case and assumes that it does not matter so much to the electrons what the nuclei do and vice versa. Or one can consider the nuclear spin without taking into account the nuclear vibrations and vice versa. Each of the sub-systems contributes to the total energy and the following equation lists the most important contributions [CD]:

$$E_{\text{total}} = E_{\text{electronic}} + E_{\text{vibration}} + E_{\text{rotation}} + E_{\text{electron spin orientation}} + E_{\text{nuclear spin orientation}} + E_{\text{translation}}$$

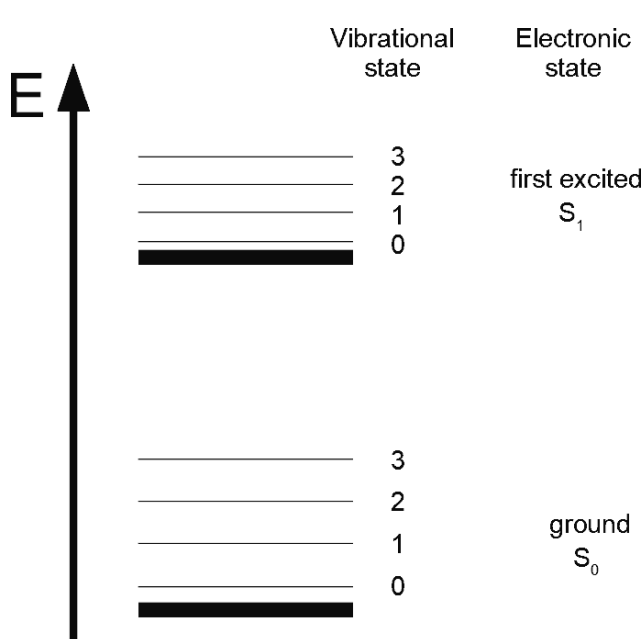
We have: the energy of the electrons in their orbitals ($E_{\text{electronic}}$), the energy due to the vibrations of the atoms ($E_{\text{vibration}}$), the energy of molecular rotations (E_{rotation}), the energy due to the orientation of the spins of the electrons ($E_{\text{electron spin orientation}}$), the energy due to the orientation of the spins of the nuclei ($E_{\text{nuclear spin orientation}}$), and the energy due to the translational movement of the molecule in space ($E_{\text{translation}}$), in other words, the thermal energy.

In the above equation, the energy contributions are listed according to the separation between energy levels. Electronic levels have the largest gaps between them and translational levels the smallest.

In order for a transition to occur for example from a lower to a higher energy level, energy must be provided. This energy might come from thermal energy but also from the absorption of a photon. The former means that higher energy levels are populated at higher temperatures (see below). The latter means that the photon energy has to match the energy gap between two energy levels. This is one of the fundamental rules (Bohr frequency rule, see below) of spectroscopy. Because the gaps are different for different sub-systems, different photon

energies are needed to study different subsystems. For example high energy photons (UV / visible light) are used to study electronic transitions whereas low energy photons are needed for nuclear spin transitions (radio waves). In turn, the spectral range determines the technical implementation of the experiment, as different materials and different approaches are needed for different spectral ranges, for example to guide and detect radiation.

EXAMPLE: ELECTRONIC AND VIBRATIONAL ENERGY LEVELS

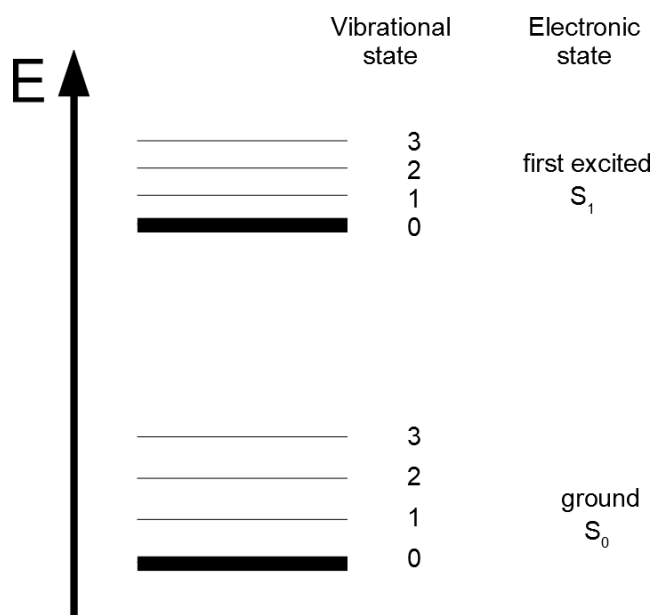


Simple representation of the electronic ground state and the first electronically excited state and of the vibrational levels

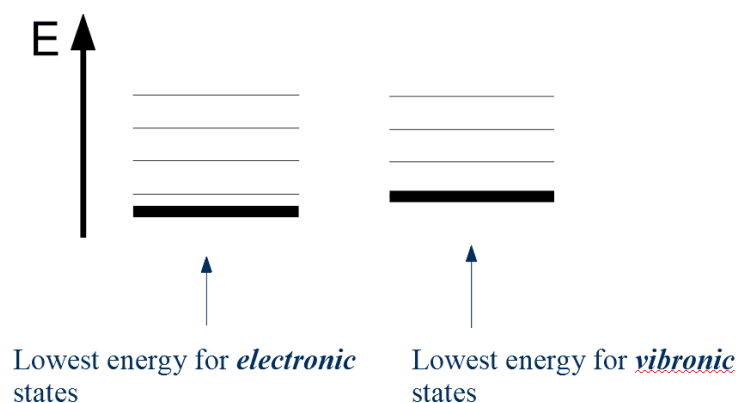
The thin lines show the total energy of the molecule in its particular electronic and vibrational state. Only the vibrational levels 0 to 3 are shown, but there are many more. I did not show them in order not to make the plot too confusing. The upper bold line illustrates the energy level of the electrons in the first excited state and the vibrational levels belonging to the electronically excited state are depicted above. As we will see in the vibrational spectroscopy lecture, there are many nuclear vibrations and each of them has its own ladder of energy levels.

We will now discuss energy levels at the example of electronic energy levels and vibrational energy levels, which we will encounter in the next few lectures. The electronic energy is the sum of the energies of the electronic orbitals and the vibrational energy is the sum of the energies of all nuclear vibrations. The figure on the left shows a simple representation of the energy levels of the electronic ground state and the first electronically excited state and of the vibrational levels in these states. The vertical axis is the total energy of the molecule.

The bold lines consider only the energy of the electrons, whereas the thin lines consider the total energy of electrons and nuclei together. The bold line on the bottom is drawn for the electronic ground state and the thin lines indicate a few of the vibrational levels which belong to the electronic ground state.



In the above illustration, the electronic ground state energy is shown separately from the ground state energy of electrons and nuclear vibrations together. However, in many illustrations these two are combined as shown on the left. Here, the bold line illustrates the energy of the electrons in the electronic ground state plus the energy of the nuclear vibrations in their ground state. In other words, it illustrates the total energy of the molecule in the ground state. The upper bold line illustrates the energy in the electronically excited state and in the vibrational ground state of the electronically excited state. When one considers both the electronic states and the vibrational states together one uses the technical term *vibronic state*.



The difference between these two ways of illustrating energy levels is shown on the left. On the left hand side, we have the case where we consider the electronic and vibrational energy levels separately. The bold line illustrates the energy of the electronic ground state and ignores the energy of the nuclei. The lowest possible total energy is given by the energy of the electrons in their ground state plus the energy of the vibrations in their ground state. This energy corresponds to the lowest thin line.

On the right hand side, the energy levels of vibronic states are plotted. Here the bold line corresponds to the lowest energy of electrons and nuclear vibrations together. Thus, it illustrates the ground state energy of the molecule and its energy equals that of the lowest thin line on the left hand side.

You can recognize which type of illustration is used by looking at the spacing between the bold line and the first vibrational level shown. On the left side, this spacing is much smaller than the spacing between the subsequent vibrational levels, whereas on the right hand side the spacing to the first vibrational level shown is the same as that to subsequent levels.

When the first spacing is smaller than the other spacings, then the first thin line corresponds to the vibrational ground state because the energy of the vibrational ground state is just half of the energy difference to the next vibrational states. In contrast, when the first spacing is the same as the other spacings, as on the right hand side,

the energy of the vibronic states is shown. Then the bold line illustrates the lowest possible energy of the system.

POPULATION OF ENERGY LEVELS

As mentioned above, thermal energy can raise the energy from that of the ground state to that of an excited state. This depends on the temperature (higher temperature = more thermal energy) and the energy gap between the ground and the excited state. A smaller gap means that less thermal energy is needed to populate (occupy) the excited state. The relative occupancy of two states with different energies is given by the Boltzmann distribution:

$$n_{\text{upper}}/n_{\text{lower}} = \exp(-\Delta E/kT)$$

where n_{upper} is the number of molecules in the higher energy state, n_{lower} is the number in the lower energy state, ΔE is the energy gap between the two states, and k is the Boltzmann constant (1.38×10^{-23} J/K). A special case of the Boltzmann distribution is that calculated for 1 mol of molecules. In that case k has to be replaced by R , the gas constant ($R = 8.31$ J mol⁻¹ K⁻¹, $R = N_A k$). Remember that $RT = 2.5$ kJ/mol at room temperature.

When ΔE is small or T is large ($\Delta E \ll kT$), $\exp(-\Delta E/kT)$ approaches e^0 , which is 1. The number of molecules in the upper and lower levels is then equal. In the opposite case (ΔE large or T small, i.e. $\Delta E \gg kT$), the exponent is very large and $\exp(-\Delta E/kT)$ very small. Then only the ground state is occupied. [CD]

As mentioned above, the energy gaps between adjacent energy levels depend on the sub-system considered. For example electronic spin states are very closely spaced, nuclear spin states are even closer. This implies that excited states are considerably populated at room temperature. For nuclear spin states, the populations of excited state and ground state are nearly equal with only 1 out of 20 000 spins more in the ground state [Ja]. In contrast, different electronic orbitals have often quite large separations between their energy levels. As a consequence, only the ground state is populated at room temperature. Intermediate between these extremes are the energy gaps between nuclear vibrations. The gaps between energy levels of the rapidly oscillating vibrations is still larger than the thermal energy, meaning that most molecules are in the vibrational ground state. However, the gaps of slow vibrations are comparable to the thermal energy meaning that a considerable number of molecules is in vibrationally excited states.



Population of energy levels. Left: larger energy gap between ground state and excited state means that the ground state is more populated than the excited state. Right: small energy gap with the consequence that ground and excited state are nearly equally populated. The usage of large and small means large and small energy gaps compared to the thermal energy kT .



Interaction of electromagnetic radiation with matter

THE MASS ON A SPRING MODEL

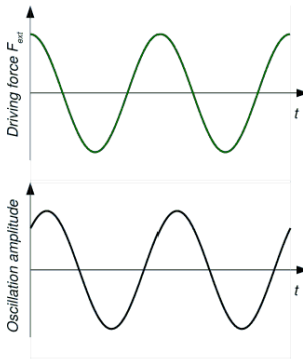
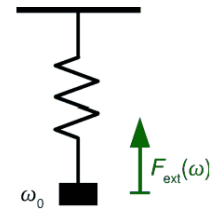
We will now discuss the interaction between electromagnetic radiation and matter and start with a simple classical model for this interaction before discussing the different phenomena that take place when matter interacts with electromagnetic radiation.

Our classical model is a mass on a spring that is subjected to an oscillating driving force and to damping, which is another word for friction. We will apply this model to describe the interaction between electromagnetic radiation and matter when we discuss the different spectroscopic methods in the following lectures. Then it will be important to identify what is the driving force and what corresponds to the mass on a spring. In most cases the driving force corresponds to the electric field of the radiation and the mass to the electrons or to the nuclei in the interacting molecules.

Analogy

Driven, damped mass on a spring

Electric field drives movement of electrons or nuclei



Driving force, ω
Electric field

Steady state displacement, ω not ω_0

Phase difference when friction
Interaction with the environment

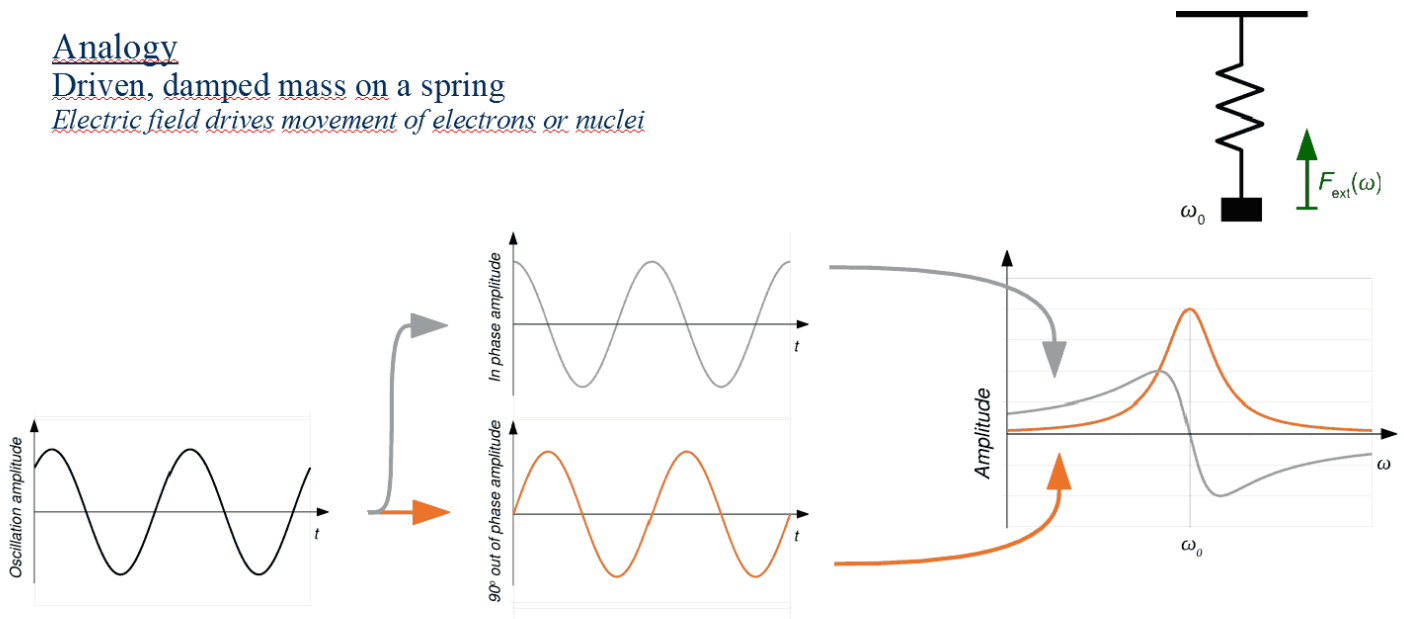
The mass on a spring model with driving force (green) and movement of the mass (black) indicated.

When a spring with attached weight is extended and released, it will oscillate at its intrinsic frequency ω_0 which is determined by the spring force constant. However, when it is driven by a sinusoidally time-varying driving force, $F_0 \cos \omega t$, it will eventually oscillate with the external frequency ω (the frequency of light). When there is friction (= interaction with the environment), then the spring does not immediately follow the driving force, it lags behind. One says that there is a phase delay between driving force and spring movement. The phase delay occurs because friction/resistance has to be overcome. One can separate the resulting spring movement in a part that is in phase (0 or 180° phase difference between spring and driving force) and one which is 90° out of phase. The amplitude of these two components depends on the driving frequency and on the intrinsic frequency of the spring (panel e). The components are called dispersion and absorption because their frequency dependencies resemble those for the refractive index and the absorption of energy by the spring respectively. We are interested in the absorption curve in the following.

Analogy

Driven, damped mass on a spring

Electric field drives movement of electrons or nuclei



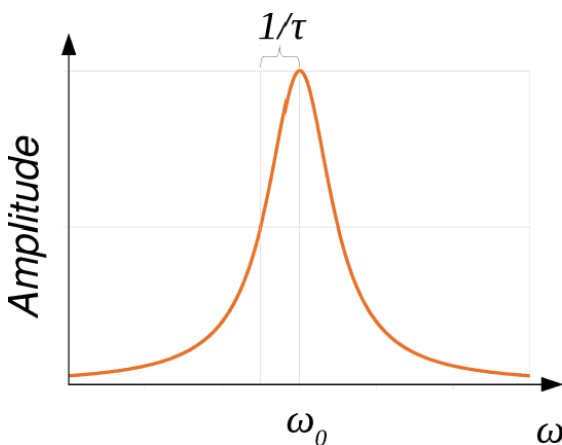
Amplitudes of in-phase and out-of-phase components depend on driving force frequency ω .

Decomposition of the mass movement into an in-phase and an out-of-phase movement.

The curve for x'' (absorption) has Lorentzian line shape

$$x''(\omega) = \tau [1 + (\omega_0 - \omega)^2 \tau^2]^{-1}$$

where ω is the angular frequency of the driving force, ω_0 the intrinsic angular frequency of the weight on the spring in the absence of driving force and τ the relaxation time which is inversely proportional to the friction coefficient.



A Lorentz curve.

The interpretation of the curve for x'' is as follows: the amplitude of the oscillation that is 90° out of phase to the driving force is maximal when the frequency of the driving force matches the intrinsic or natural frequency of the weight on a spring (= frequency of oscillation in the absence of a driving force). This increase in amplitude can be quite dramatic. It is only possible by energy transfer from the driving force to the spring and it turns out that the energy absorbed by the spring is largest when the two frequencies match. Such a phenomenon is called **resonance**. It gives evidence for the absorption of energy by the spring from the driving force. In spectroscopy, this corresponds to energy absorbed by the atoms or molecules from the electromagnetic radiation.

Why is it the 90° out of phase component of the movement that is enhanced when the driving force frequency equals the intrinsic frequency? The 90 degrees out of phase amplitude lags behind the driving force. Thus the driving force is in phase with the velocity of the mass. It generates the strongest acceleration when the velocity is highest. The maximum velocity increases and therefore also the amplitude of the oscillation. Because the intrinsic frequency of the spring and the frequency of the driving force are the same, the driving force tends to increase the velocity throughout the entire oscillation. The only exceptions are the turning points, where velocity and force are zero. Eventually this would lead to an infinite amplitude, but because there is always friction opposing the movement, the amplitude stays finite.

Speaking about friction, friction is also a force that is proportional to velocity, but it is always directed against the velocity. If a spring is extended and oscillates freely but is subjected to friction, then the oscillation amplitude decreases with time. This is just the opposite of what we just discussed for the driven oscillator. When the driving force has the same direction as the velocity at all times, then the amplitude of the oscillation increases.

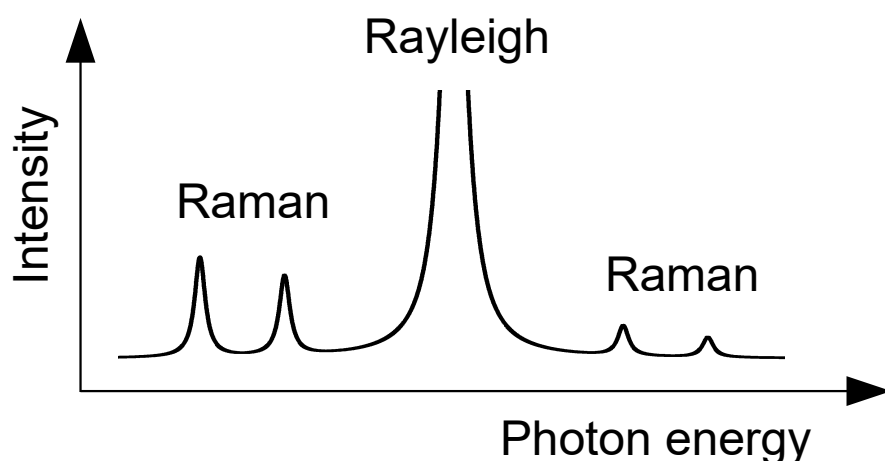
The width of the Lorentz curve depends on the relaxation time. The name relaxation rate stems from the properties of the system in the absence of an external, oscillating driving force. When the spring is extended, it undergoes oscillations which decrease in amplitude with time because of friction. This is called relaxation back to the equilibrium position. The decay time τ for the oscillation amplitude is called relaxation time. It depends on the friction, because strong friction leads to a fast decay. In more general terms, interactions with the environment determine the band width of the absorption curve. This is true for the oscillating spring but also for electronic absorption. Strong interaction with the environment gives short life times of the excited states (= short relaxation time, i.e. the time for return to the ground state) and this produces broad absorption bands.

INTERACTION PHENOMENA

When light interacts with matter, several phenomena can take place. Their analysis yields information on the biological system under investigation. The phenomena are:

Scattering

Matter scatters electromagnetic waves, i.e. changes their direction of propagation. Scattering might occur elastically (Rayleigh scattering) or inelastically (one example is Raman scattering, after Sir Raman, an Indian scientist who discovered the effect), which means with or without energy difference of scattered and incident light.

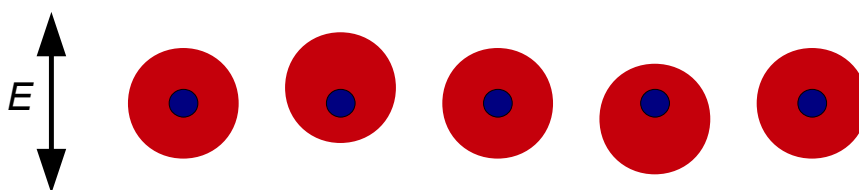


The Figure on the left shows a spectrum of scattered light. The horizontal axis can be wavelength, wavenumber or frequency. The vertical axis is the intensity of scattered light. The large majority of photons is scattered elastically (Rayleigh scattering, after the English physicist Lord Rayleigh for his discovery of the effect, pronounced ray-lea like in *X-ray* and in *leave*).

An everyday example for scattering is the following: the sky is blue because it is the blue light that is preferentially scattered from small particles in the atmosphere. Thus we see blue light when we do not directly look into the sun. Milk or an oil-water suspension are turbid because light is scattered from small oil droplets.

Scattering can be explained as follows: the electric field vector of the electromagnetic wave induces oscillations of the electrons. This creates an oscillating dipole because the nuclei are much less moved because of their larger mass. The oscillation dipole acts like a small antenna and emits radiation in all directions.

In the figure below, the oscillating electric field vector is shown and its effect on an atom at different times (phases) of the oscillation. The electron cloud is shown in red and the nuclei in blue. The nuclei are nearly stationary because of their large mass, but the electron cloud moves. Whenever the position of the center of the electron cloud is different from that of the nucleus, a dipole moment is generated. This oscillates with time and emits radiation.

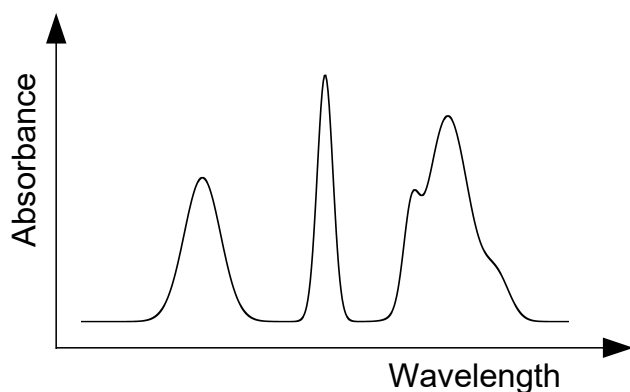


If the molecules have a permanent dipole moment an additional effect takes place: the molecules will reorient under the influence of the electric field which again gives rise to an oscillating dipole moment.

The emitted radiation from a molecule superimposes with that of all other molecules and with the incident radiation. Therefore, scattering is also the basis of the phenomena reflection and refraction: reflected light is the superposition of the scattered light from all molecules, and refraction is the result of the superposition of the incident wave with all scattered waves.

Absorption

Matter absorbs energy from the electromagnetic wave in specific regions of the electromagnetic spectrum. Leaves are green because light of other colors is absorbed, green light is transmitted and reflected preferentially.



The Figure on the left shows an absorption spectrum. The horizontal axis can be wavelength, wavenumber or frequency. The vertical axis is absorbance (see below for the definition of this quantity). The peaks are referred to as absorption bands or absorption lines. Some of the bands are well resolved (the band at the left and in the middle) whereas the bands on the right overlap. The left and the center band of the right band profile are still resolved, but the small component band on the right of the center band is not resolved. It generates a shoulder.

Emission

Light can be emitted for example from a hot body (light bulb) or after light of one wavelength has been absorbed. In the latter case, the emitted light has lower energy (longer wavelength) than the absorbed light. Example: Whiter than white effect of washing powders: light in the ultraviolet region of the spectrum is absorbed, but emitted is light in the visible region which makes the T-shirt look brighter.

Photochemistry

Visible and UV light have enough energy to alter a molecule, for example to break covalent bonds, to induce rotations around bonds or to ionize the molecule by abstraction of an electron or a proton. These effects are very important in biology (photosynthesis).

Transitions between energy levels*SPECTROSCOPY IS APPLIED QUANTUM MECHANICS*

Spectroscopy is an experimental method which aims at obtaining molecular information on the system under study. The link between observation and information is provided by the theory of the molecular interaction between electromagnetic or particle radiation and matter. In general, this interaction perturbs atoms and molecules which often makes them lose or gain energy. The theoretical challenge is to describe the extent of these effects and why this happens only at certain wavelengths.

This can be best done in the framework of quantum mechanics. Fundamental will be the description of energy levels of a molecule and of the interaction between radiation and matter. The following is a summary of important concepts in quantum mechanics. [CS]

1. The state of a system (e.g., an atom, a molecule, a crystal) is described by a *wavefunction* (in the position representation of the general theory), named for example Ψ (greek letter psi), or by a *state vector* $|\Psi\rangle$ in a multi-dimensional vector space - the Hilbert space. Expressing quantum mechanics in terms of wavefunction or state vector are two equivalent forms of the theory and we will use both.

2. A quantity that can be observed in an experiment (energy, dipole moment, location in space, velocity, spin, etc.) is represented by an *operator*. A famous example is the Hamilton operator \mathbf{H} for the energy. For experts: bold print of " \mathbf{H} " indicates that the symbol represents an operator. An operator acts on state vectors and alters them. For example $\mathbf{H}|\Psi\rangle$ represents the effect of an energy measurement on the state vector $|\Psi\rangle$. The result is a new state vector that is in general different from $|\Psi\rangle$.

3. When an experiment is repeated (like measurement of position or energy), the result is in general not the same. Either the experimental values can adopt several discrete values (like when the energy of a molecule is measured or they are continuous and scatter around an average value (like when the position of a particle is measured). This "irreproducibility" is an inherent property of the quantum mechanical theory. What is reproducible is the average result of the measurement. This average value can be computed by calculating the expectation value of the operator that represents the observable being measured. For example $\langle\Psi|\mathbf{H}|\Psi\rangle$ is the expectation value for the energy. $\langle\Psi|\mathbf{H}|\Psi\rangle$ is the scalar product between two vectors, $\langle\Psi|$ and $\mathbf{H}|\Psi\rangle$ where the latter one is the vector that is obtained after \mathbf{H} operates on $|\Psi\rangle$. The scalar product is zero when the two vectors are orthogonal (90° angle), it is maximum when the vectors are parallel.

Representing the state of a system by wavefunctions, the expectation value is written $\int\Psi^*\mathbf{H}\Psi\,d\tau$ where Ψ^* is the conjugate complex of Ψ and the integration is over all space. If an experiment always gives the same result, then the system is in an eigenstate of the operator that represents the observable being measured. For example, if an energy measurement always gives the same result, then the system is in an energy eigenstate. Formally this is written in the following way $\mathbf{H}|\Psi_e\rangle = E|\Psi_e\rangle$ and an energy measurement gives $\langle\Psi_e|\mathbf{H}|\Psi_e\rangle = E\langle\Psi_e|\Psi_e\rangle = E$ (because the eigenvectors are normalized according to $\langle\Psi_e|\Psi_e\rangle = 1$). E is a energy eigenvalue of \mathbf{H} . This means the following: when the Hamilton operator acts on the eigenstate $|\Psi_e\rangle$ the result is the same eigenstate $|\Psi_e\rangle$ times the energy E of the system. Therefore, the system is in the same state before and after the energy measurement. If the system is in an eigenstate of the Hamilton operator (= an energy eigenstate), then it remains in that state for indefinite times. One says that the state is stationary, i.e. does not change with time. No transitions to other states are possible.

Let us consider a system that is described by the Hamilton operator \mathbf{H} with two energy eigenstates $|\Psi_0\rangle$ and $|\Psi_1\rangle$. Because they are energy eigenstates, they are stationary, i.e. the probability of the finding the system in one of these states does not change in time. This is easily shown using the Schrödinger equation

$$i\hbar\,d\Psi/dt = \mathbf{H}\Psi$$

which gives for either $|\Psi_0\rangle$ or $|\Psi_1\rangle$ (calculated for Ψ_0)

$$i\hbar\,d\Psi_0/dt = E_0\Psi_0$$

with E_0 being the energy of state Ψ_0 . The solution of this differential equation is

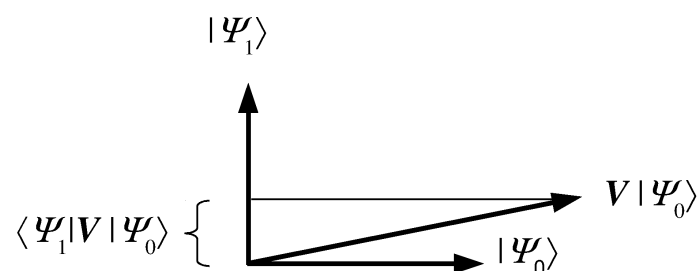
$$\Psi(t) = \Psi(0)\exp(-iEt/\hbar)$$

The corresponding probability $\Psi^*(t)\Psi(t)$ is time independent:

$$\Psi^*(t)\Psi(t) = \Psi^*(0)\Psi(0)$$

So, if the system is in state $|\Psi_0\rangle$ at a given time, it will remain in that state for all times and no transitions are possible. However, transitions between energy eigenstates are fundamental to understand spectroscopic experiments. How does this fit together?

4. In order to induce transitions, the system needs to be perturbed. The system can for example be a molecule. The Hamilton operator of the unperturbed molecule describes the energy contributions and interactions within the molecule, i.e. it does not consider interactions with the environment. A perturbation is for example the interaction of the molecule with an electromagnetic wave. This interaction changes the energy of the system, which means that the original Hamilton operator is no longer a "good" operator to describe the energy of the molecule. Instead the Hamilton operator of the perturbed system consists of the original Hamilton operator H and a perturbation operator V . Since H is no longer an appropriate Hamilton operator, its eigenstates (i. e. the eigenstates of H) are no longer proper eigenstates of the perturbed molecule. Still they are in many cases good approximations of the real eigenstates and it is easier for us to use them also in the description of the perturbed molecule. If we do so, the perturbation induces transitions between the eigenstates of the unperturbed Hamilton operator, for example between the eigenstates $|\Psi_0\rangle$ and $|\Psi_1\rangle$ considered above. The perturbation V might induce transitions from $|\Psi_0\rangle$ to $|\Psi_1\rangle$. According to *Fermi's golden rule*, the probability for the transition is proportional to $|\langle\Psi_1|V|\Psi_0\rangle|^2$. In the position representation this scalar product is written as $|\int\Psi_1^*V\Psi_0d\tau|^2$ where the integration is over all space.



An interpretation is easiest in the Dirac notation: The scalar product (or dot product) $\langle\Psi_1|V|\Psi_0\rangle$ is a projection of vector $V|\Psi_0\rangle$ on vector $|\Psi_1\rangle$ as shown in the figure on the left. It analyses how similar these two vectors are. The projection is zero, if the two vectors are orthogonal, it is maximal if they have the same direction. An analogy is the calculation of the y -component (or y -coordinate) of a vector in 3 dimensional space. This is the same as the projection

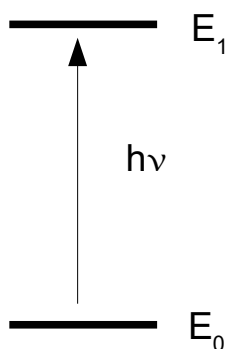
of this vector on the y -axis. If the perturbation V has no influence on $|\Psi_0\rangle$ then $V|\Psi_0\rangle = |\Psi_0\rangle$ and $\langle\Psi_1|V|\Psi_0\rangle = \langle\Psi_1|\Psi_0\rangle = 0$ since both vectors are eigenvectors of the Hamilton operator and therefore orthogonal to each other. Therefore, a transition will only occur, if the perturbation makes the initial state $|\Psi_0\rangle$ somewhat similar to the final state $|\Psi_1\rangle$, i.e. if $V|\Psi_0\rangle$ is rotated away from $|\Psi_0\rangle$ towards $|\Psi_1\rangle$.

Summary: transitions between energy eigenstates can only be induced by a perturbation of the system (molecule). The probability of a transition depends on the degree to which the perturbation distorts the initial state of the system so that it becomes more similar to the state after the transition. When we discuss spectroscopic methods, it will be important to identify the perturbation and the relevant eigenstates of the system. We will leave these key concepts for now but discuss them in more detail when we discuss the individual spectroscopic methods.

ABSORPTION

Bohr frequency rule

Electromagnetic radiation can induce transitions between energy levels. For example, energy from the electromagnetic wave can be absorbed which takes the system to a state with higher energy. But the system is selective: only radiation with a particular wavelength can induce the transition and can therefore be absorbed.



Transition from the ground state E_0 to an excited state induced by absorption of electromagnetic radiation.

To understand this selectivity, we have to move from the wave picture for electromagnetic radiation to the particle picture. The transition can take place if the energy of the photons matches the energy gap ΔE between the energy levels.

$$\Delta E = h\nu$$

This is known as the **Bohr frequency rule** and applies to many different types of spectroscopic experiments.

The Bohr frequency rule $\Delta E = h\nu$ is a consequence of the corpuscular theory of light. Light with low frequency (long wavelength) can only excite low energy transitions, no matter how large the intensity (= number of photons) is.

Selection rules

Apart from the Bohr frequency rule, other rules exist that are particular for the transitions induced in the experiment. We will encounter them when we discuss transitions between particular energy levels. These rules are called *selection rules* and they classify transitions into "allowed" and "forbidden" transitions. However, these categories are not absolute. In practice, an allowed transition is one which has a high probability, a forbidden transition is one with a low probability.

Definition of selection rule according to IUPAC Gold book: "A rule that states whether a given transition is allowed or forbidden, on the basis of the symmetry or spin of the wavefunctions of the initial and final states." (<https://goldbook.iupac.org/S05549.html>)

The reason for the failure of a clear cut distinction between allowed and forbidden transitions is a deficiency of the theory applied to derive the selection rules. The theory often is based on a simplified system which is only an approximation of the real system.

An example for such a simplification is the assumption that the phase of the electromagnetic wave is the same across the entire molecule (Douglas, Burrows, Evans, Chapter 1 in Applied Photochemistry edited by Evans 2013).

Different ranges of electromagnetic radiation probe different molecular properties

We have seen above that the gaps between energy levels can be very large or very small depending on the sub-system. The gaps can be overcome by absorbing a photon with an energy that matches the energy gap between

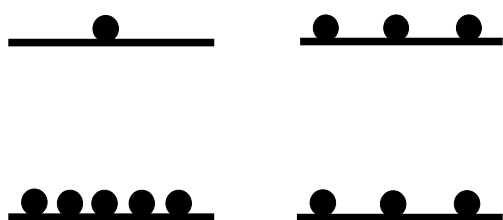
energy levels. Thus photons with very different energies are needed to induce transitions in the different sub-systems, or in other words to probe different properties of the system.

It was already mentioned that the energy gaps between different electronic orbitals can be large which means that photons of light in the UV or visible spectral range are needed to induce them. The energy gaps between vibrational levels are smaller, therefore electromagnetic radiation in the infrared spectral range is sufficient to induce the respective transitions. Even smaller are the gaps between electronic spin states which means that microwave radiation induces the transitions. Gaps between nuclear spin levels are even smaller and therefore radiowaves are sufficient to induce transitions.

Absorption depends on the population of energy levels

Electromagnetic radiation can induce transitions from the ground state to an excited state. However, it can induce also a transition from an excited state back to the ground state. This is called stimulated emission. The total number of absorption and emission processes is given by the transition probability times the number of molecules in the ground or the excited state. The transition probability is the same for absorption and emission when the same excited state is involved in both cases. When nearly all molecules are in the ground state, there are therefore more absorption than emission processes and energy is absorbed by the molecules from the electromagnetic radiation. If however, the number of molecules in an excited state is the same as in the ground state, then the number of absorption and emission processes is the same and there is no net absorption of energy.

In some experiments (small difference in occupancy of ground and excited states, high power radiation) the occupancy of energy levels can significantly change during the experiment. First, most molecules are in the ground state. Electromagnetic radiation induces then mostly transitions to the excited state and energy is absorbed by the system. If the intensity is high (many photons), more and more molecules adopt the excited state. When the populations of excited and ground state are equal, then there is no net absorption of energy any more. This phenomenon is called *saturation*. [CD]



Population of energy levels. Left: the ground state is more populated than the excited state. Absorption can occur. Right: absorption of electromagnetic radiation has made the population of the excited state as large as that of the ground state. No net absorption can occur.

Absorption depends on concentration

Not surprisingly, the amount of absorbed energy depends on the number of absorbing molecules, i.e. on their concentration. (The following is adapted from [CS])

Let us now consider a simple absorption experiment: The incident light has intensity I_0 at wavelength λ . It traverses a sample with a path length d . The light that is not absorbed by the sample emerges with intensity I_1 .

Consider a sample of molecules in a layer perpendicular to the direction of light propagation, and sufficiently thin (dx) so that the light intensity within this layer is essentially constant. Then the fraction of light absorbed ($-dI/I$) should be simply proportional to the number of absorbing molecules. The resulting equation is

$$-dI/I = C \varepsilon' dx$$

where C is the concentration of absorbing molecules and ε' is a proportionality constant that is proportional to the probability for an individual molecule to absorb a photon.

If we integrate this equation over the entire sample (integrating the left-hand side from initial intensity I_0 to final intensity I_1 , and the right-hand side from zero to d we obtain

$$\ln(I_0/I_1) = -\ln(I_1/I_0) = C \varepsilon' d$$

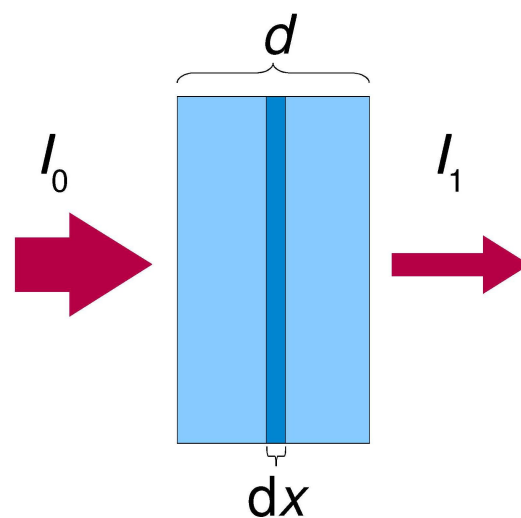
Converting to log base 10, we have the common form of the **Beer-Lambert law** (also known as Beer's law or the Lambert-Beer law or the Beer-Lambert-Bouguer law after those who independently discovered the law in various forms: Bouguer in 1729, Lambert in 1760, and Beer in 1852 [wikipedia.org].):

$$A(\lambda) = \log(I_0/I_1) = -\log(I_1/I_0) = C \varepsilon(\lambda) d$$

where $\varepsilon = \varepsilon'/2.303$ is called the **molar absorption coefficient** and A is called the **absorbance** or (sometimes) the **optical density OD**.

$\varepsilon(\lambda)$ is a molecular property that depends on the wavelength of the incoming light. It is proportional to the probability of inducing a transition at that particular wavelength. It was previously called **extinction coefficient** and this term is still widely used. The reason, why it is recommended to switch from extinction coefficient to absorption coefficient is that extinction includes not only absorption but also intensity losses due to scattering. If only absorption occurs then extinction and absorption are the same. Often only the absorption coefficient (ε_{\max}) at the wavelength of maximal extinction (λ_{\max}) is of interest. It is used for example to determine the concentration of biomolecules. The value ε_{\max} for electronic transitions of typical chromophores varies over a wide range from as little as $1 \text{ M}^{-1}\text{cm}^{-1}$ to more than $10^5 \text{ M}^{-1}\text{cm}^{-1}$. ε_{\max} depends also on the molecular sub-system. For example, the absorption coefficient for vibrational transitions is typically a factor of 100-1000 lower than that of electronic transitions.

ε can be related to the probability of an individual absorption process (best in [CS]). This probability is abbreviated B and named Einstein coefficient. The Einstein coefficient B is the transition rate per unit energy density of the radiation. The loss of intensity dI (=



loss of energy) will be proportional to B , the energy of the absorbed photons $h\nu$ and the number of incident photons which is proportional to incident intensity. Thus:

$$-dI \sim \nu B I$$

Since $-dI = C \epsilon' I dx$ we get the following proportionality for ϵ :

$$\epsilon \sim \nu B \quad \text{or} \quad B \sim \epsilon/\nu$$

This proportionality holds for a very sharp absorption band only. Real absorption bands have a certain band width $\Delta\nu$ (full width at half maximum, see below). Then we have to replace ϵ/ν by the integral over $d\nu$. This can be approximated by $\epsilon_{\max} \Delta\nu/\nu_{\max}$ (index "max" indicates values taken at maximum absorption) assuming that the band width is much smaller than the frequency which makes the frequency essentially constant across the absorption band and by approximating $\int \epsilon d\nu$ by $\epsilon_{\max} \Delta\nu$. This gives:

$$B \sim \epsilon_{\max} \Delta\nu / \nu_{\max}$$

The quantity A is called the **absorbance** or (sometimes) the **optical density**. Note that "absorption" denotes the physical process and "absorbance" is the quantity that describes how much light is absorbed. One can say both "*absorption spectrum*" and "*absorbance spectrum*" but not "the *absorption* is 0.5" (correct is "the *absorbance* is 0.5") or "*absorbance* is a process which attenuates the incoming light" (correct: "*absorption* is a process which attenuates the incoming light"). Absorbance is a quantity without unit. Some spectroscopy programs label the vertical axis of a spectrum with "absorbance (a.u.)" meaning "absorbance in arbitrary units". This is nonsense. Absorbance has no units, not even arbitrary ones. Most useful is the expression

$$A(\lambda) = -\log(I_1/I_0)$$

because it expressed absorbance in terms of the fraction of incident light that reaches the detector. Note that the relationship is logarithmic. $A = 2$ means that only 1% of the incoming light transmits the sample and reaches the detector. When $A = 3$, the transmitted light intensity is 10 fold less, i.e. only 0.1% of the incoming intensity. Somewhere here is the limit for accurate measurements. Higher absorbance values mean that even less light reaches the detector which makes accurate measurements difficult. Equally difficult are measurements at low concentrations. When only very little light is absorbed, the small difference of light intensity with and without sample is difficult to measure. Therefore, the absorption of single molecules cannot be measured. Typical concentrations are in the μM range.

RELAXATION

Definition

Systems in excited states have the tendency to return to the ground state. A transition from an excited state to the ground state is called relaxation. One speaks of relaxation processes or says that the system relaxes back to the ground state. Relaxation can involve the emission of a photon or not.

Non-radiative relaxation processes [CD]

If relaxation does not lead to the emission of a photon one speaks of non-radiative relaxation. We distinguish two non-radiative relaxation processes: **thermal relaxation** and **resonance energy transfer**.

Thermal relaxation: Here, a molecule loses energy by collisions with other molecules, which transfer energy from one molecule to another. This increases the kinetic energy of many molecules, which means that the temperature of the sample increases.

Resonance energy transfer: Here the relaxation of one molecule is coupled to the excitation of another molecule. This energy transfer takes place without emission of a photon and is therefore a non-radiative relaxation process. It is most efficient when the transition energies of the two molecules are the same, i.e. when they are in resonance (like two tuning forks with the same pitch). Resonance energy transfer is important in fluorescence spectroscopy.

Spontaneous emission

In spontaneous emission an excited state relaxes by spontaneous emission of a photon without interaction with the environment. According to "ordinary" quantum mechanics, spontaneous emission is not allowed because the excited state is an eigenstate of the system and therefore stationary, i.e. in the absence of a perturbation the system would remain in the excited state forever. To explain spontaneous emission, the electromagnetic field needs to be quantized (quantum electrodynamics or QED). The consequence is, that the excited state of the molecule is no longer an eigenstate of the whole system (molecule plus electromagnetic field) and therefore that it has a finite life-time. When the molecule relaxes to its ground state, the electromagnetic field converts from its ground state to an excited state in which it contains one photon [Wikipedia, spontaneous emission]. The probability for spontaneous emission is strongly dependent on the energy difference between excited state and ground state, expressed in terms of frequency ν of the emitted photon. [CD]

$$A \sim B \nu^3$$

where A is an Einstein coefficient that describes the probability (= rate) of spontaneous emission (do not confuse with absorbance A) and B the Einstein coefficient for induced emission and (induced) absorption. Both are proportional to the absorption coefficient ε . Thus the probability for spontaneous emission is proportional to the probability of absorption. The stronger the absorption of a substance, the higher the probability for spontaneous emission and therefore the shorter the lifetime of the excited energy level.

The following proportionality for A holds approximately [Sy]:

$$A \sim \nu_{\text{peak}}^2 \Delta\nu \varepsilon_{\text{max}}$$

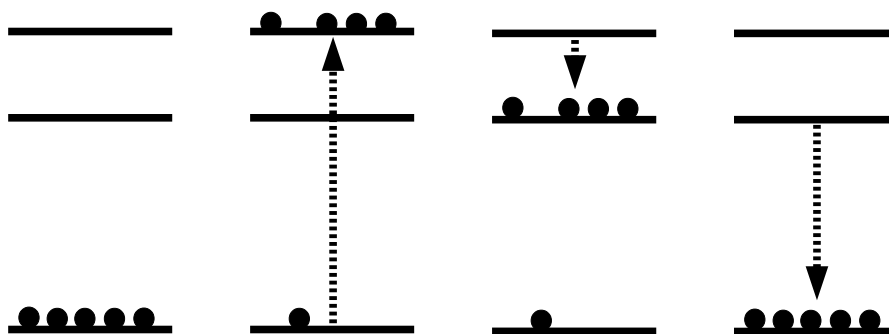
where ν is the frequency, $\Delta\nu$ is the half width and ε_{max} the maximum absorption coefficient. Spontaneous emission is therefore the more probable compared to induced emission and absorption, the larger the energy difference between the two energy levels.

Two particular kinds of spontaneous emission are fluorescence and phosphorescence. We will discuss them later in more detail.

Stimulated emission [CD]

Stimulated emission (or induced emission) is the emission of a photon induced by interaction with electromagnetic radiation. For example, an incoming photon stimulates the transition from an excited state to the ground state. The incoming photon has an energy that matches the transition energy [A p434]. The transition creates a further photon with the same, energy, phase and direction as the incoming photon. Therefore radiation generated by stimulated emission is coherent to the incoming radiation. The probability for stimulated emission is the same as for absorption (at least for simple cases like a two-state system, which we have described by the Einstein coefficient B).

Stimulated emission generates the light emitted by lasers (light amplification by stimulated emission of radiation). In a laser, a higher occupancy of an excited state is generated first. Spontaneous emission generates photons which stimulate the relaxation of other molecules, i.e. generate more photons which stimulate further relaxation processes and so on. This continues until the excited state is depleted.

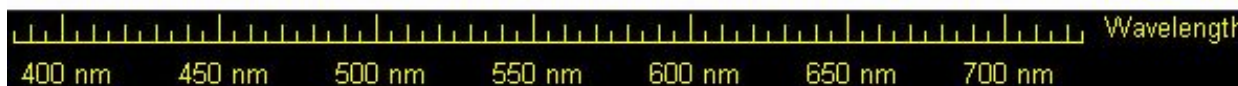


Principle of the laser at the example of a three-state system. From left to right. (1) Initial state: all molecules are in the ground state. (2) Pumping excites the system to the highest energy state. (3) From there it relaxes via non-radiative decay to an intermediate energy level - the laser level. The lifetime of this level is high, therefore the population of this level becomes higher than that of the ground state. (4) Radiation stimulates emission of the laser light from this level. □

LINE SHAPE AND LINE WIDTH

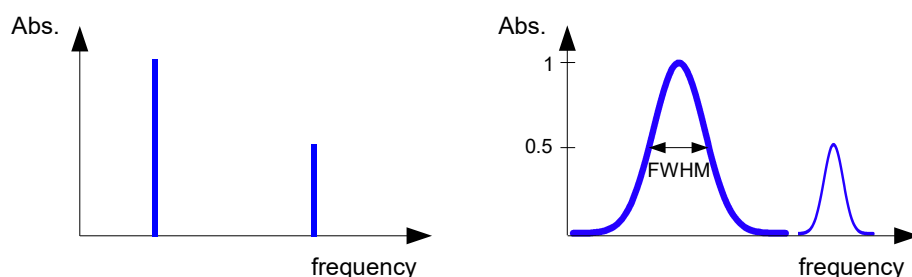
Where does the word "line" come from?

The words line shape and line width are used to describe the shape of an absorption band and its width. The word "line" stems from the observation of relatively narrow bands in emission spectra of compounds that were heated in flames. The high temperature made that several excited states were occupied which relaxed under emission of photons. The color of the emitted light corresponded to the energy of the transition [H]. Even when the bands in a spectrum are broad we can describe them by using the expressions "line shape" and "line width", although "band shape" and "band width" are also used.



Line spectrum of helium [Nasa]

A broad absorption band means that the underlying transition can be excited by a range of energies not only a particular energy. There are several reasons for this: the energy gap is not exactly defined out of quantum mechanical reasons and different molecules may have slightly different energy gaps because they have different environments or otherwise different properties. Therefore a range of energies can be absorbed and a plot of absorbance versus frequency gives absorbance bands with a given width instead of narrow lines. This plot is called a spectrum. We will characterize line width by their width at half of the maximum absorption, either by the full width at half maximum (FWHM) or the half width at half maximum (HWHM).



Instead of infinitely sharp lines (left) one observes absorbance bands. FWHM is the full width at half maximum.

Absorption is therefore not only characterized by its strength and the frequency of absorbed radiation but also by the shape and width of the absorption band. We will discuss first the shape of absorption bands and then the width. Emission experiments give also lines which are associated with a line shape and a line width. Therefore the following discussion also holds for emission spectra.

Homogeneous and inhomogeneous line broadening

The line width of a transition depends on a number of factors. We distinguish homogeneous and inhomogeneous line broadening. **Homogeneous broadening** broadens the spectrum of a single molecule. The underlying mechanism acts on all molecules in the same way. A *Lorentzian line shape* function describes homogeneously broadened absorption or emission bands.

Inhomogeneous broadening is the result of ensemble effects when the effects on different molecules are not equal. As a result, different molecules will have different energy gaps between ground and excited state. The absorption and emission bands under such conditions are described by a *Gaussian line shape* function. This line shape can be thought as a superposition of many individual Lorentzian lines from the individual molecules.

Lorentzian line shape

A Lorentzian line shape describes the behaviour of an oscillating system that is subjected to an external oscillating driving force and some form of damping. A simple mechanical analogue is a mass on a spring that experiences friction. But a system like this describes very generally the interaction between electromagnetic radiation and matter, for example the induced movement of electrons in molecules which gives rise to

electronic transitions and the induced movement of nuclei giving rise to vibrational transitions. The Lorentzian line shape $L(\omega)$ is given by

$$L(\omega) = \tau [1 + (\omega_0 - \omega)^2 \tau^2]^{-1}$$

where ω is the angular frequency of the incident radiation, ω_0 the natural (or resonance) angular frequency of the system in the absence of external forces and τ the relaxation time. For a spring, it is inversely proportional to the friction coefficient and describes the interaction of the system with the environment. The stronger the interaction, the shorter is the relaxation time τ . τ is also the characteristic time constant with which the extended spring returns to the equilibrium extension in the absence of a driving force. In spectroscopy τ describes the life time of an excited state. τ in the Lorentzian line shape function determines how broad this function is. To be more specific, the inverse of τ (τ^{-1}) is the half width at half maximum (HWHM) of a Lorentzian absorption band. Thus the width of an absorption band is inversely related to the life time of the excited state reached in the transition. [M]

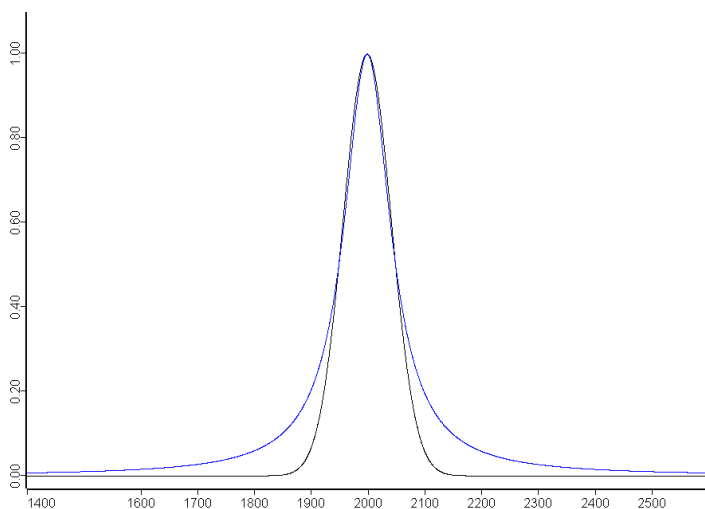
The unit of the line shape function is seconds. The line shape function needs to be multiplied with a factor to give the absorption. [M]

Gaussian Line Shape

Inhomogeneously broadened absorption bands have Gaussian line shapes. The line shape function has the same form as the Gaussian normal distribution used for the statistical scattering of measured values around an average value. This is of course no coincidence but comes from the statistical distribution of energy gaps around an average value. A Gaussian function is an $\exp(-x^2)$ function and a useful function for spectroscopy is:

$$G(\omega) = A \exp [- (\Delta\omega/\Delta\omega_{1/2})^2 \ln 2]$$

where A is a prefactor that is of no interest for us, $\Delta\omega$ is $\omega_0 - \omega$ and $\Delta\omega_{1/2}$ the half width at half maximum. $\hbar \omega_0$ is the average transition energy.



Comparison of a Gauss curve (black) and a Lorentz curve (blue) which have both the same HWHM of 50 units. The Lorentz curve is a bit narrower around ω_0 but much broader far away from ω_0 [generated with OPUS].

Natural line width

In the absence of interactions between a molecule or an atom with the surroundings, the line width of a transition is called natural line width. Even under these conditions, spectral lines are not infinitely sharp. This is due to the Heisenberg uncertainty principle, which says that a state's energy and its life time cannot both be determined with infinite precision. In other words, the energy of a state with a finite life time τ is uncertain by ΔE with

$$\Delta E \tau \geq \hbar \quad \text{or} \quad \Delta \nu \tau \geq 1/2\pi$$

To specify the energy of a state exactly ($\Delta E = 0$) requires that the molecule in that state has an infinitely long lifetime. Since no molecule in an excited state has an infinite lifetime, it follows that no excited state has a precisely defined energy and that spectral lines have a finite width. This is called lifetime broadening. Short-lived energy states give rise to broad spectral lines, while long-lived states give narrower spectral lines. [CD]

The ground state has an infinite life time (Theoretical Chemistry and Physics of Heavy and Superheavy Elements, U. Kaldor, S. Wilson, p119; Physnet MISN-0-241, The uncertainty relations: description, applications, P. Signell) in the absence of interactions since it cannot undergo a spontaneous transition to a higher energy level. Therefore it has no energy uncertainty. Even in the presence of electromagnetic radiation, leading to induced absorption, its life time is longer than that of an excited state. This is because the excited state has two mechanisms to relax, via induced emission with the same probability as induced absorption but additionally via spontaneous emission. Therefore the natural line width is determined by the excited state energy uncertainty.

We consider now only spontaneous emission as a relaxation path. We know that the probability of spontaneous emission is proportional to the probability of absorption and to ν^3 . Because the probability is the inverse of the lifetime we see that the line width follows the same dependencies. Therefore transitions with a large energy gap and strong absorption give broad bands, those with small energy gap and weak absorption narrow bands.

Natural line broadening is an example for homogeneous line broadening because all molecules are affected in the same way. The line width is described by a Lorentzian line shape. The broadening effect of the limited life time is usually very small compared to other causes of broadening. [H]

Pressure Broadening

Pressure broadening is a form of lifetime broadening. When particles collide there is an exchange of energy which decreases the lifetime of an excited state. The denser the particles are packed, the more likely are collisions and the broader will be the line of a transition. This broadening is usually homogeneous and produces a Lorentzian line shape in most cases. [H]

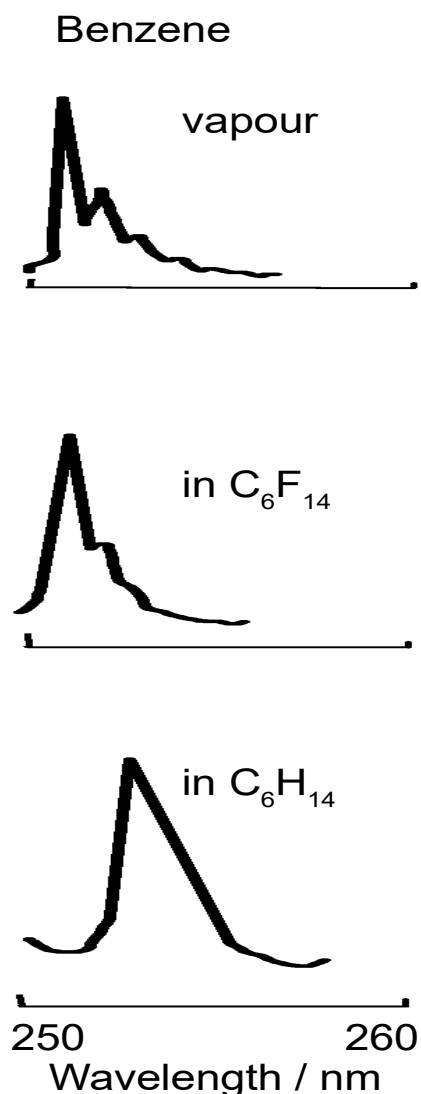
Doppler broadening [H]

The frequency of electromagnetic radiation that a molecule "feels" depends on its velocity because of the Doppler effect. The same effect makes the sound of an approaching ambulance of higher pitch (frequency), that

of a disappearing ambulance of lower pitch. Let particles at rest have a transition with an energy gap of $\Delta E = h\nu_0$. This transition will be observed when the frequency of electromagnetic radiation is ν_0 and when the particles are at rest. If a particle moves away from the source of electromagnetic radiation, the frequency "felt" by the particle is lower than if it is at rest. To excite the transition, the frequency of the emitted radiation has therefore to be higher than ν_0 . If it moves towards the source, the frequency of radiation has to be lower. Therefore the frequency ν at which a given transition is observed depends on the speed s of the particle and is given by

$$\nu = \nu_0 / (1 \pm s/c) \quad \text{and} \quad \Delta\nu \approx \nu_0 s/c$$

where c is the speed of light. Because the speed of the particles are distributed according to the Maxwell distribution (for a gas), there is a spread of velocities which broadens the line of a transition. This line broadening is usually far greater than the natural line width. The broadening is inhomogeneous, since not all particles behave in the same way (they have different velocities) and this results in a Gaussian line shape.

Environmental Broadening

The environment of a molecule can affect the energy gap between two energy levels. Individual molecules in an ensemble of molecules interact often slightly different with the environment which gives rise to a spread of transition energies. Each single molecule gives rise to a Lorentzian line shape. But because of the spread of transition energies, the central frequencies differ. If all the Lorentz lines of the different molecules are summed up the resultant line shape is Gaussian. The line broadening is inhomogeneous.

An example for broadening because of solute solvent interactions. The benzene spectrum in gas phase shows fine structure that is lost in solvent. [redrawn by C. Baronio from CS]

Overview over Spectroscopic Methods*SPECTRAL REGIONS*

The range of wavelengths used in spectroscopy of biological molecules is impressive. The photon energies range from millions of kJ/mole (more than sufficient to break the strongest covalent chemical bonds if the energy could be localized) to less than 10^{-3} kJ/mole (far smaller than typical thermal energies: RT is about 2.5 kJ/mole at room temperature). [CS] In the high energy regime of the electromagnetic spectrum (γ -rays, X-rays, UV/visible radiation) the photons have enough energy to break bonds and destroy molecules. Such reactions are called photoreactions. They are not always destructing, for example one of the most important processes on this

planet, photosynthesis, is initiated by a photoreaction where an electron is abstracted from a molecule. In this case, the photoreaction is caused by light close to the low energy end of the visible spectral range. Lower energy radiation is more gentle to molecules and does not harm them. [HS]

Table. Biologically useful spectroscopic regions. [CS] Electrons are also listed, although they are of course not electromagnetic radiation.

<i>Typical wavelength</i>	<i>Approx. energy / kJ mol⁻¹</i>	<i>Spectral region of measurement</i>	<i>Phenomenon observed</i>	<i>Techniques and applications</i>
4 pm	10 ¹⁰	particle wave	Scattering, absorption	Electron microscopy
0.1 nm	10 ⁶	X-ray	Scattering	X-ray diffraction
300 nm	4 × 10 ²	Ultraviolet (UV)	Electronic transitions Vibrational transitions	UV-spectroscopy, fluorescence, circular dichroism (CD) Raman spectroscopy
<i>Carbon-carbon bond energy 360 kJ/mol</i>				
600 nm	2 × 10 ²	Visible (vis)	Electronic transitions Vibrational transitions	vis-spectroscopy, fluorescence, circular dichroism (CD) Raman spectroscopy
10 μm	10	Infrared (IR)	Vibrational transitions	Infrared spectroscopy
<i>Thermal energy RT at room temperature 2.5 kJ/mol</i>				
1 cm	10 ⁻²	Microwave	electron spin transitions	Electron paramagnetic resonance (EPR)
10 cm	10 ⁻³	Radio frequency	nuclear spin transitions	Nuclear magnetic resonance (NMR)

USEFULNESS

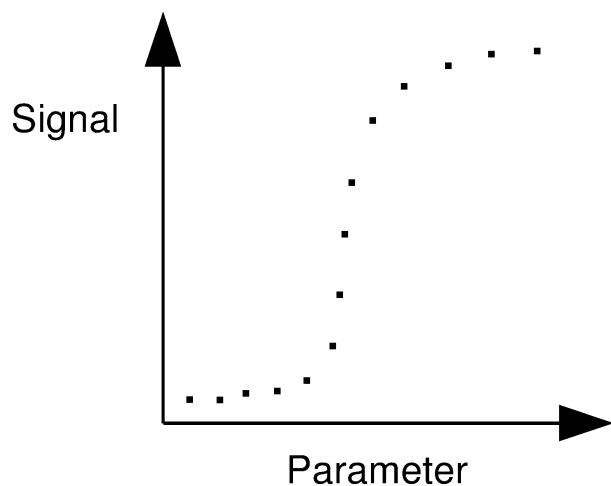
All methods have their advantages and disadvantages. None tells us all what we want to know about the biological system. Each gives a specific answer to a specific question. With the answers of several methods we can start to build up the jigsaw puzzle that represents our knowledge about the biological system.

Some of the methods look at the whole molecule, some only at some part of it. This part is either intrinsic or has been introduced artificially by the researcher. Some are able to investigate the system close to physiological conditions, others (like x-ray crystallography and electron microscopy) not.

Table: Information provided and effort needed of several biophysical methods. Information is listed under benefits and is divided into information on structure (from little to much information: *s*, *S*, **S**) and dynamics (*d*, *D*, **D**). Effort is listed under pain and measured in terms of time that is needed for one experiment and money for the equipment.

<i>Method</i>	<i>Benefits</i>	<i>Pain</i>
Electron microscopy	S,D	P
X-ray diffraction, scattering	S,D	P
Electronic absorption spectroscopy	<i>s,D</i>	<i>p</i>
Fluorescence	<i>S,D</i>	<i>p</i>
Circular dichroism (CD)	<i>S</i>	<i>P</i>
Raman spectroscopy	<i>S</i>	<i>P</i>
Infrared spectroscopy	<i>S,D</i>	<i>P</i>
Electron paramagnetic resonance (EPR)	<i>S,D</i>	<i>P</i>
Nuclear magnetic resonance (NMR)	S,D	P

MONITORING CHANGES



Monitoring changes with spectroscopy. A spectroscopic signal from a reporter group is monitored in dependence of a parameter like temperature, concentration, etc.

Some methods give a lot of direct information on the biomolecule of interest. Examples are NMR and X-ray crystallography which can reveal the 3D structure of biomolecules. Other methods are often used to monitor changes. The only requirement is a spectral probe that reports on an event like protein folding or unfolding, DNA melting, protein conformational changes, binding of ligands, etc.. This probe or reporter group can be an intrinsic group or can be introduced artificially. Examples for techniques used in this way are absorption spectroscopy, fluorescence, circular dichroism and infrared spectroscopy.

Using spectroscopy to monitor changes is a very powerful concept. It allows researchers to investigate the many parameters that affect the event of interest. Thus, a curve like that on the left side gives access to a lot of important information.

Example ligand binding

A reporter group gives a stronger (weaker) signal when a ligand binds. From this it can be determined how strong a ligand binds. From the equilibrium constant the energetics of the interactions (ΔG) can be inferred. Different ligands can be compared and in that way parts of the ligand that are important in binding determined. Mutants can be used to identify protein residues important in binding. It can be studied whether ligands bind to the same binding site or not, whether binding is affected by other parameters like temperature, pH value etc. Detecting ligand binding is important for drug development. Here, binding of molecules to a protein target is tested for a very large number of molecules.

Example protein folding

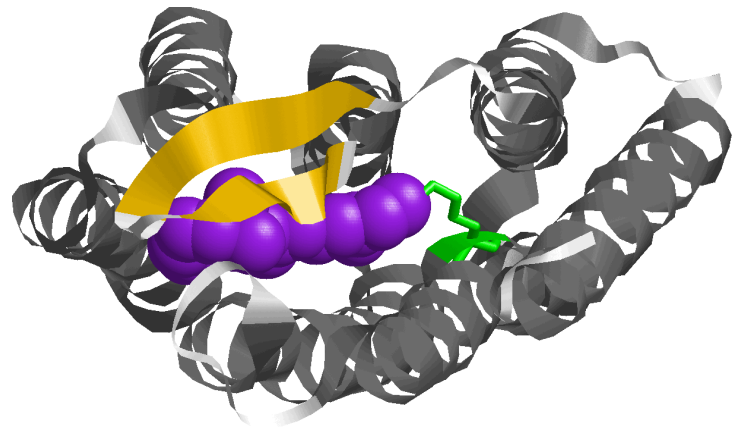
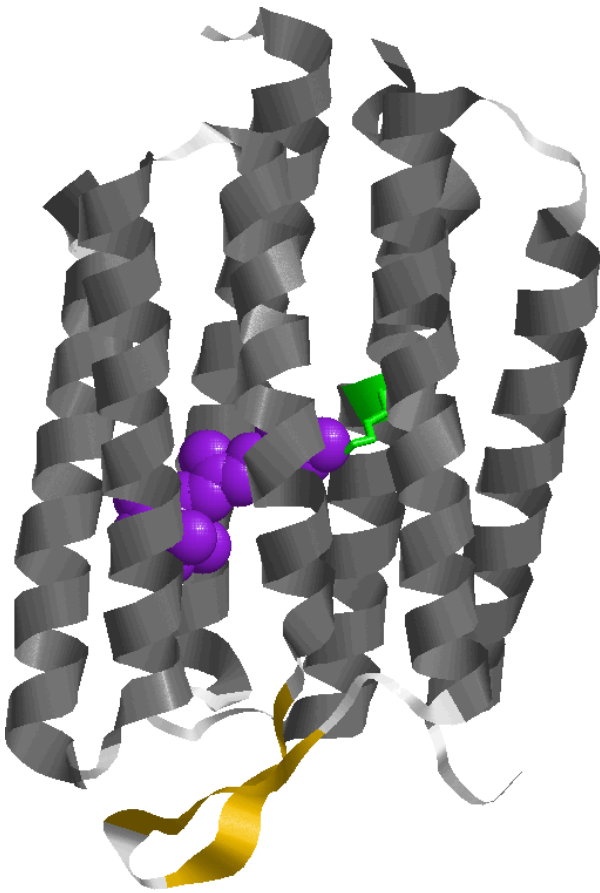
A reporter group has a different signal when the protein is folded. Stability of the protein under a variety of conditions can be probed for example by determining the temperature at which it unfolds. Protein folding can be followed in a time-resolved way. Then the kinetics of folding can be investigated, whether folding is a cooperative process or whether it proceeds in a number of steps, how folding is influenced by mutations that abolish specific residue-residue interactions in the native structure etc.

Bacteriorhodopsin

As an example where spectroscopy in the visible spectral range made important contributions to the understanding of a protein, we will discuss bacteriorhodopsin. It is a small integral membrane protein which functions as a light-driven proton pump: it converts light energy into a transmembrane proton gradient. The proton gradient is converted by the ATP-synthase into ATP. Bacteriorhodopsin is found in the cell membrane of salt-loving microorganisms (archaea). It provides them with an emergency generator of energy when the oxygen concentration is low and respiration ceases.

Bacteriorhodopsin is one of the proteins which are most intensely studied for several reasons: (i) it is a model for an important class of membrane bound receptors - the G-protein coupled receptors. (ii) It is an example for a membrane transporter, which are essential for all organisms, and generates a 10,000-fold difference in proton concentration on either side of the membrane. (iii) It is "handy". The transport process can be easily triggered by light, which enables many kinds of experiments, it is easily obtained in large quantities and unusually stable.

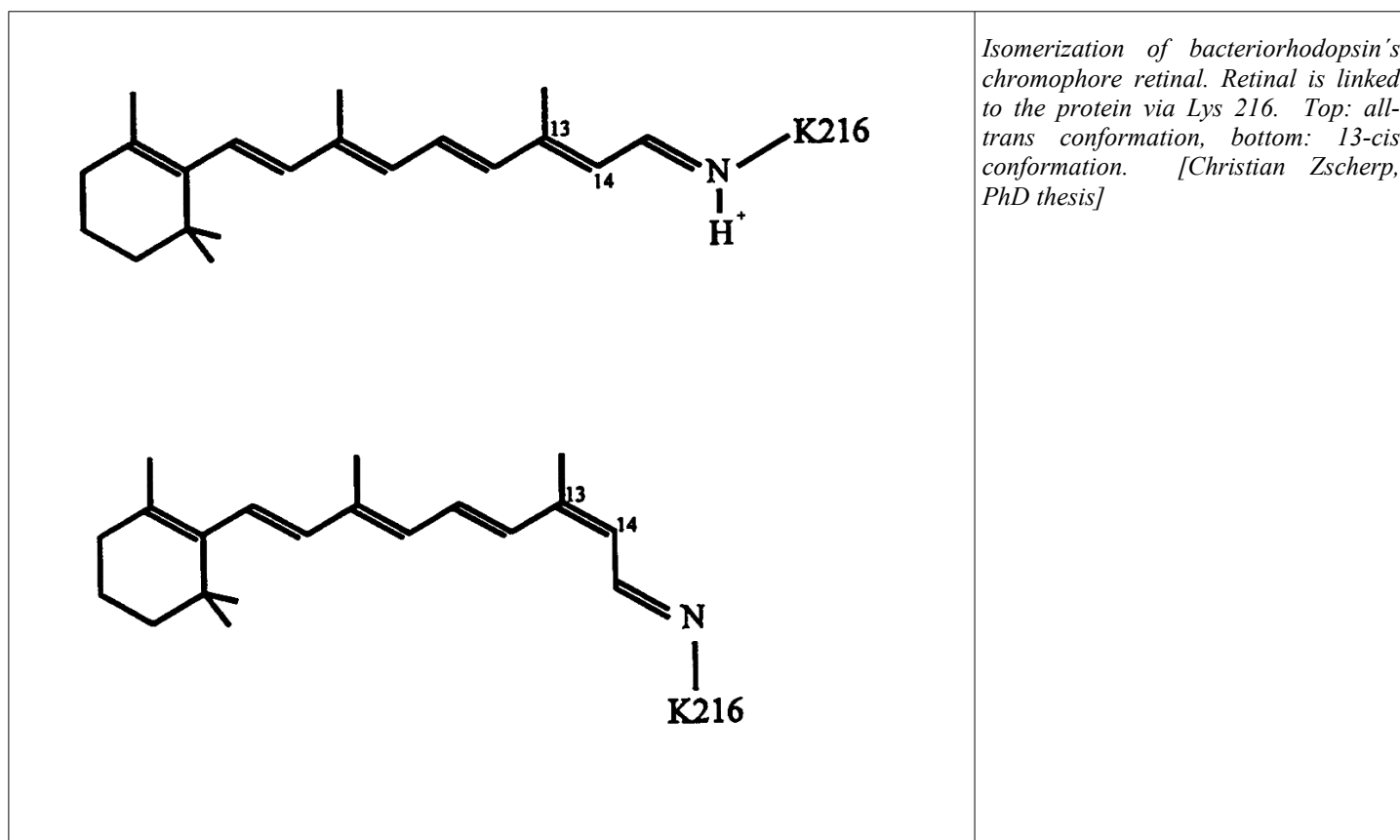
Bacteriorhodopsin consists of seven transmembrane helices and a chromophore ("color bringer", i.e. a molecule that absorbs visible light) called retinal. The retinal forms a covalent bond with a Lys residue in helix G (this functional group is then called *Schiff* base). [K] The chromophore retinal is also present in our retina in the protein rhodopsin which is the primary light receptor and is related to bacteriorhodopsin.



Bacteriorhodopsin model. Protein helices are shown in grey, β -strands in yellow, retinal is shown in an all atom representation (purple), and Lys 216 is green. Shown are two views. Left: parallel to the membran surface. The membran runs approximately horizontally. Right: along the membrane normal [Figure made with Rastop and IMOL.pdb].

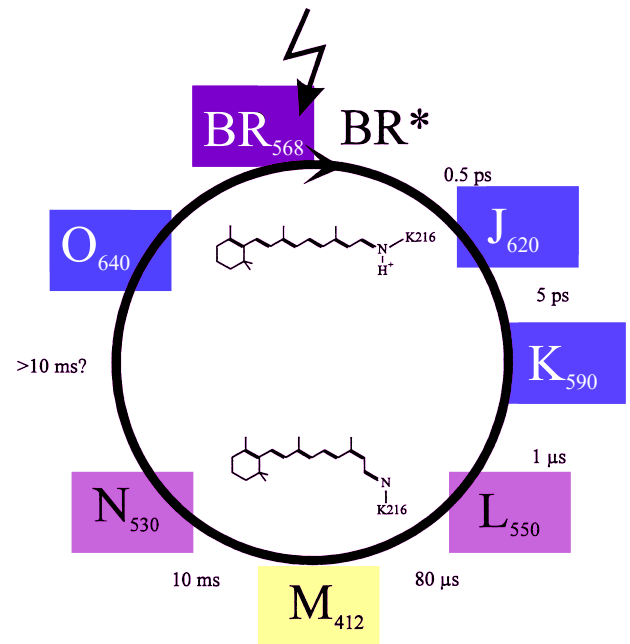
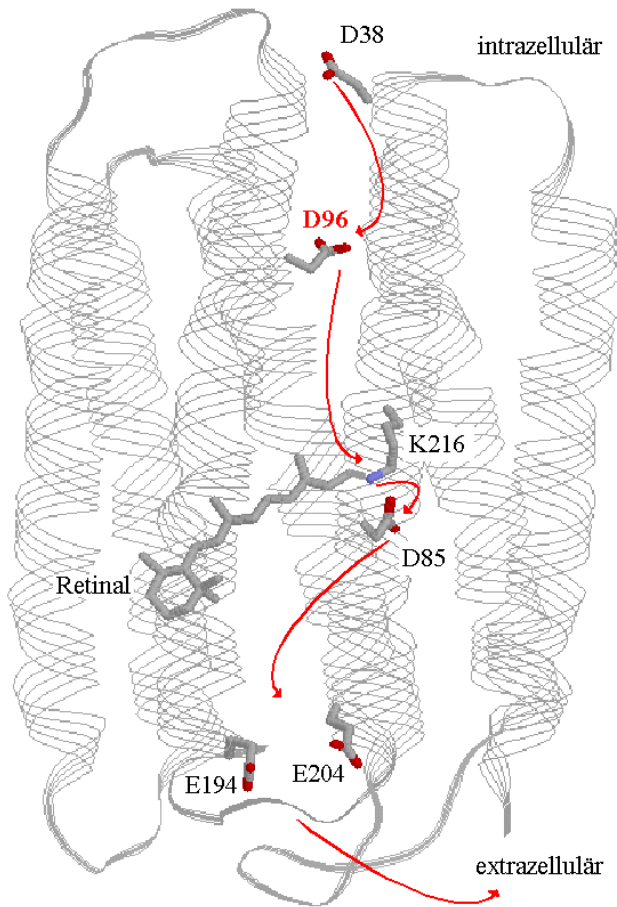
After absorption of a photon, retinal in bacteriorhodopsin changes its configuration from all-*trans* to 13-*cis* as shown here. This is a rotation around a double bond. Interestingly, such a rotation is also possible in the ground state. It takes place when BR turns from the light-adapted state where it is in all-*trans* configuration to the dark-adapted state which is a 1:1 or 2:1 mixture of the 13-*cis* and all-*trans* isomers (Oesterhelt et al. 1973, Pettei et al. 1977, Scherrer et al. 1989). The isomerization is catalysed by the protein (Sperling et al. 1977).

The photoinduced all-*trans* to 13-*cis* isomerization is facilitated in the excited state because of a changed electron density distribution in the molecule. This isomerization is an example for a photoreaction. The photoisomerization reaction in bacteriorhodopsin proceeds with high yield (~60% [Haupts et al. Annu. Rev. Biophys. 1999; Hasson et al. PNAS 93 (1996) 15124-15129]). This is in contrast to model studies in methanol, where retinal linked to a protonated Schiff base forms *cis* isomers after light excitation only with a probability of 15%. The by far dominating conformer in methanol is the 11-*cis* conformer [Hasson et al. PNAS 93 (1996) 15124-15129]. Thus the protein environment increases the yield of the photochemical reaction and determines the conformation of the photoproduct.



Isomerization of retinal in bacteriorhodopsin is transmitted to the protein environment and starts a cascade of reaction steps which transport one proton across the membrane. This involves changes in protein structure as well as protonation and deprotonation of several groups (including D85, D96, and the Schiff base which links retinal to K216).

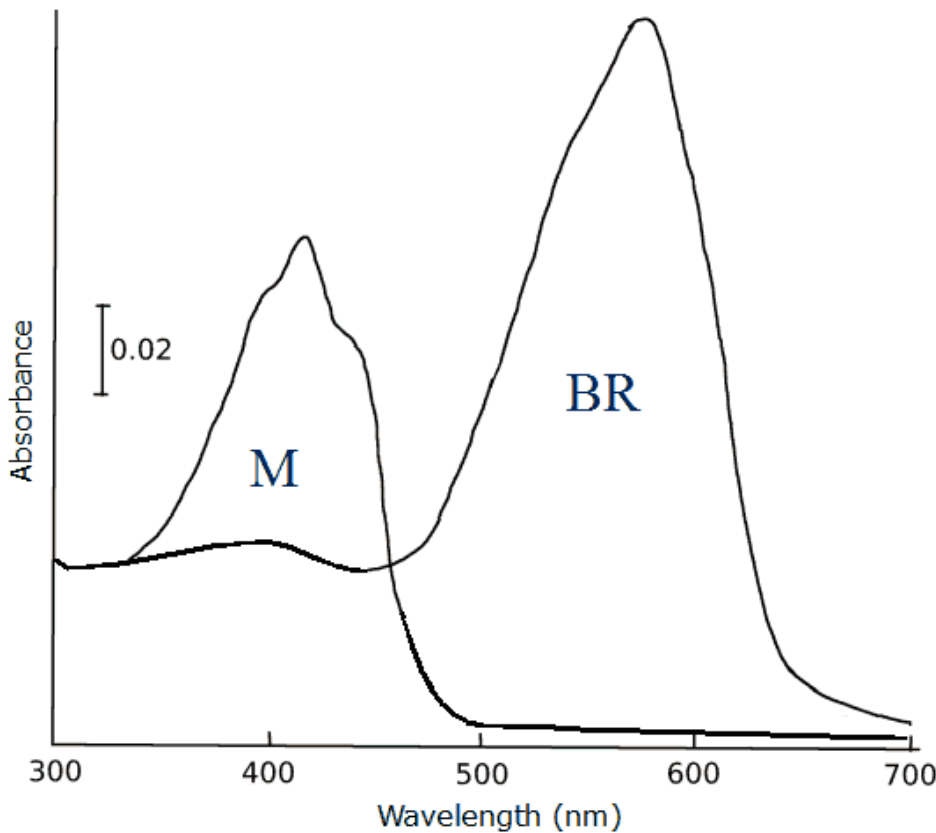
The photoreaction proceeds via several intermediate states named BR (ground state), J, K, L, M, N, and O. The intermediates have different colors, in other words, they have different absorption spectra in the visible spectral range. The indices in the figure of the reaction cycle indicate the absorbance maximum in nm. Therefore the intermediates can be followed by time-resolved spectroscopy and that has been done intensively. The color of the chromophore retinal is tuned by electrostatic interactions [Oesterhelt, *Curr. Op. Struct. Biol.* 1998, 8, 489-500]. So the protein environment determines the color of the chromophore. In bacteriorhodopsin studies, researchers take advantage of this effect to study the protein. In our retina, nature uses the same effect to enable color vision: three different types of rhodopsin proteins with the same chromophore retinal have their absorbance maximum in the blue, red and green spectral region because they provide a different environment for retinal.



Photocycle of bacteriorhodopsin. Intermediates are designated by capitals, the indices indicate the absorbance maximum in nm. In O, BR and BR the retinal is in the all-trans conformation, in all other states it is in the 13-cis conformation. The Schiff base is deprotonated only in M. [Christian Zscherp, PhD thesis, colors: inspired by Hirai T, Subramaniam S - PLoS ONE (2009)]*

Left figure. Proton transport in bacteriorhodopsin (courtesy of Christian Zscherp). The reaction cycle consists of the following steps:

- 1.) Isomerization of the retinal from all-trans to 13-cis in 0.5ps (BR→J).
- 2.) Proton moves from the Schiff base C=(NH)⁺-Lys216 to Asp85 because the pK_a of the Schiffbase decreases by 7 units upon retinal isomerization and the pK_a of Asp85 increases to more than 10.5. A proton from Glu194 or Glu204 acidifies the extracellular medium. (L→M)
- 3.) A proton moves from Asp96 via water molecules to the Schiff base, because the pK_a of Asp96 has dropped from >11 in the ground state to 7.1 in the N intermediate. Relatively large conformational changes (M→N) accompany this step.
- 4.) Asp96 reprotonates with a proton from amongst others Asp38. Retinal isomerizes back to the all-trans state (N→O).
- 5.) Proton moves from Asp85 via a proton channel to Glu194 or Glu204 (O→BR).



Left: Absorbance spectrum of the purple membrane which consists to 75% of bacteriorhodopsin and to 25% of lipids.

Right: Bacteriorhodopsin spectrum in the darkadapted ground state (1:1 mixture of all-trans and 13-cis retinal, absorbance maximum at 560 nm) and in the M intermediate. [redrawn by C. Baronio from Oesterhelt, Angew Chem. Int. Ed. Engl. 15 (1976) 17-24 and extended according to Oesterhelt 1976 and Zimányi et al 1999]

BR absorbs in the visible spectral range, which is entirely due to the chromophore retinal. The rest of the protein absorbs only in the ultraviolet, not in the visible spectral range. The absorbance maximum in the ground state is near 570 nm. Yellow and green light is absorbed while blue and some red light can pass. Therefore the color of the BR ground state is purple (spectrum of BR is extended according to the other spectrum in Oesterhelt 1976).

Retinal in solution absorbs at about 380 nm, and model retinal compounds with protonated Schiff base absorb at 440 nm. The additional red shift to 568 nm for the all-*trans* chromophore, originates from electrostatic interactions and from the planarity of the retinal backbone [Lanyi, J. Struct. Biol. 1998, 124, 164-178]. This is another example for the influence of the protein environment on the color of the chromophore. It defines the electrostatic interactions and restricts the conformation of the chromophore.

The largest spectral change in the photocycle occurs when the M intermediate is formed. The M intermediate absorbs at 150 nm lower wavelength than the ground state and has an absorbance maximum near 400 nm. Therefore it has little absorption in the visible spectral range and is nearly white. But not quite, because some of the blue light is absorbed. This makes the M intermediate appear pale yellow. (spectrum of and of the M intermediate extended according to Zimányi PNAS 1999 and <http://group.szbk.u-szeged.hu/ormosgroup/abs/abs.html>).

These spectral differences can be exploited to study the photocycle of bacteriorhodopsin. For example, one can find out how fast the M intermediate forms. For this one measures the absorbance at 400 nm for example.

Such a measurement will show an increase in absorbance when the M intermediate forms. By repeating such measurements at different wavelengths, optical spectroscopy has detected the intermediates in the photocycle and characterized the number and kinetic properties of them. One can then continue and study the effect of mutations on the photocycle. In this way, residues can be identified that are important for particular reaction steps.

In addition, optical spectroscopy has the important role of assigning properties measured by other methods to particular steps in the photocycle. For example, time resolved infrared spectra reveal changes of retinal conformation and protonation and deprotonation of amino acid side chains. When we correlate this information with spectra in the visible spectral range, we can find out at which step of the photocycle these changes occur.

Similarly, when proton uptake from and proton release to the bulk are measured, these events can be correlated to the photocycle using the spectral properties of the BR intermediates.

Optical spectroscopy has also been used to assess the functional activity of bacteriorhodopsin in crystals, which is an important prerequisite for time-resolved X-ray crystallography.

Of particular advantage in these studies is the relative simplicity of the visible spectroscopy experiments, the high time resolution and the high sensitivity (little consumption of protein).

Finally after many years of research, the following view of the photocycle has emerged:

The first steps are absorption of a photon, isomerization of the retinal from all-trans to 13-cis and return to the electronic ground state. All this happens in 0.5ps in the transition from BR to the J intermediate. The retinal is in a strained conformation (Lanyi 2006) that relaxes somewhat in the steps to the L intermediate (Lanyi 2001).

Next, the proton moves from the Schiff base $C=(NH)^+-Lys216$ to Aspartate 85. Remember that the Schiff base is the linkage between retinal and lysine 216 of bacteriorhodopsin. The proton transfer takes place because the pKa of the Schiff base decreases by 7 units upon retinal isomerization (source?) and the pKa of Asp85 increases to more than 10.5 (Barth 2007). A proton from a protonated water cluster near Glutamate 194 and Glutamate 204 acidifies the extracellular medium. All this happens in the L to M transition.

Then, in the M to N transition, a proton moves from Asp96 to the Schiff base. This restores the original protonation state of the Schiff base. The proton transfer takes place for two reasons: first, the pKa of Asp96 has dropped from larger than 11 in the ground state to about 7.5 in the N intermediate (Barth 2007, Lanyi 2006) and is now lower than that of the Schiff base, which is about 8 (Lanyi 2006). Second, a chain of water molecules starts to form in the M state that serves as intermediate proton acceptors (Lanyi 2006).

In the N to O transition, Aspartate 96 becomes reprotonated by a proton from amongst others Aspartate 38. The retinal isomerizes back to the all-trans state.

Finally in the transition from O to the BR ground state, the proton of Aspartate 85 moves via a proton channel to the extracellular release site near Glutamate 194 or Glutamate 204.

As you can see, it is not the same proton that is transported all the way from the intracellular side to the extracellular medium in one photocycle. Instead, several protons move towards the extracellular side, each covering a fraction of the total distance in different regions of the protein.

The mechanism of this protein is just amazing. There is one energy input into the system which is the absorption of a photon. This makes the retinal isomerize which is aided and guided by the protein environment. Isomerization of the retinal leaves both the retinal and the protein environment in a strained conformation. Both try to relax to the lowest energy conformation which is the ground state. Often such relaxation processes just lead to heat transfer to the environment. Not so in bacteriorhodopsin! Its relaxation process proceeds via a number of tightly controlled steps that couple energy relaxation to the pumping a proton.

As an illustration imagine the following: an initial energy input makes you stand up. Then you relax by sinking down into a comfortable sofa and finally adopting a resting position. At the same time you do useful work. Hard to imagine? But this is exactly what bacteriorhodopsin does.

Problems and Study Questions

1. Spectroscopy observes transitions between energy levels. They have different magnitudes for the different techniques. List spectroscopic techniques according to the energy of the transitions observed from low energy transitions to high energy transitions. Note that this does not always coincide with the spectral region used for measurement. What technique is the exception (this question can only be answered after all methods have been discussed)?
2. Name processes that can take place when light interacts with molecules.
3. What is Fermi's golden rule and what is it good for?
4. Molecules have a number of properties (electronic orbits, electronic and nuclear spins, vibrations, etc.) that determine the energy of the molecule. If one wants to investigate a certain property with spectroscopy, one must usually use electromagnetic radiation in a relatively narrow wavelength range.

Why is that so?

The properties are electronic state, vibrational state, rotational state, electron spin orientation, nuclear spin orientation (and translation). Spectroscopy is based on the observation of transitions between molecular states. The spacing (energy gap) between the states of one property is usually very different from that of another property. The energy of the radiation used in spectroscopy has to match the energy gaps between the energy levels.

5. Rank the following biophysical techniques according to the photon energy used: EPR, infrared spectroscopy, NMR, UV spectroscopy, visible spectroscopy, X-ray scattering. Where do you place the thermal energy kT in your ranking?
6. Why is it important for absorption experiments whether the thermal energy kT is smaller or larger than the photon energy?
If kT is larger than the photon energy (= energy gap between ground and excited state), then the two states are nearly equally populated and the absorption is close to zero. Absorption is highest when the population difference is large. This is the case when the thermal energy is smaller than the energy gaps.
7. Write down the Beer-Lambert law, define absorbance and explain the quantities in the formula.
8. How much light reaches the detector when the absorbance is 0, 1, 2, 3 and 4. Calculate the ratio between light intensity before and after the sample.

$I/I_0 = 1, 0.1, 0.01, 0.001, 0.0001$

9. Would you trust a concentration measurement that is done at an absorbance of 4?
Not without further checks. This absorbance corresponds to only 0.01% of the initial intensity reaching the detector. It might not even be light that has gone through the sample but could be light that is scattered or reflected somewhere in the instrument.
10. The Beer-Lambert law breaks down in non-homogeneous samples.
- Discuss the effect of air bubbles. Assume that 1 or 10% of the illuminated area is transparent. The rest is filled with your sample. How do the air bubbles affect the measured absorbance of a sample if the true absorbance is 1, 2 and 3.
 - Discuss the effect of very strongly absorbing parts of the sample. This happens for example in infrared imaging of cells. In some cell cycle phases, the DNA is highly condensed, absorbing strongly in very small areas. Assume that the absorbance is 0.1 when the DNA is homogeneously distributed. Compare this to the case that the DNA is found only in 1% of the illuminated area. The rest of the area is transparent.
11. What is the difference between absorbance and absorption?
12. Explain homogeneous and inhomogeneous line broadening.
13. Which functions describe homogeneous and inhomogeneous line broadening?
14. Give examples for homogeneous line broadening
15. Give examples for inhomogeneous line broadening. Explain why you classify the broadening effects as inhomogeneous.
16. Consider the following hypothetical case. You measure the spectral absorption of a molecule that binds to a protein. The absorption depends on the interactions between molecule and protein. When you do the measurement at very low temperature (70 K) the absorption band is sharp. When you measure at room temperature, the line is broad. Why? What is this type of broadening called and what function describes the line shape at room temperature?
Temperature increase increases the flexibility of the protein. This gives rise to a larger variation in the interactions between molecule and protein. Each molecule protein complex will have different interactions leading to slightly different spectral positions of the absorption band. This is inhomogeneous line broadening and the line shape at room temperature will be described by a Gauss function.
17. Calculate photon energies for electromagnetic radiation at 200 nm (UV), 500 nm (visible) and 2000 cm^{-1} (infrared), 1 cm (microwave, EPR) and 10 cm (radiowave, NMR) in J/mol. Compare the energies to a typical single bond energy (300 to 400 kJ/mol) and to the thermal energy ($h = 6.6 \times 10^{-34}$ Js, $N_A = 6 \times 10^{23}$ mol $^{-1}$, $c = 3 \times 10^8$ m/s). What is the significance of these comparisons?
Result: 500 kJ/mol, 200 kJ/mol, 20 kJ/mol, 10 J/mol, 1 J/mol. Look up thermal energy in the script and find out why a comparison of the calculated energies with thermal energy is important.
18. In which types of spectroscopy photons with the following wavelengths used: 200 nm, 500 nm and 2000 cm^{-1} , 1 cm and 10 cm?
200 nm: UV; 500 nm, vis, Raman; 2000 cm^{-1} : IR; 1 cm: EPR; 10 cm: NMR
19. Absorption of photons causes transitions between energy levels. Calculate the ratio between the occupancy of the lower and the higher level at room temperature for radiation of the following wavelengths 200 nm, 500 nm and 2000 cm^{-1} , 1 cm and 10 cm. How does this affect the amount of absorbed energy?
 $n_{\text{upper}}/n_{\text{lower}} = 1.4 \times 10^{-87}, 1.8 \times 10^{-35}, 3.3 \times 10^{-4}, 0.996, 0.999$. See text for interpretation.