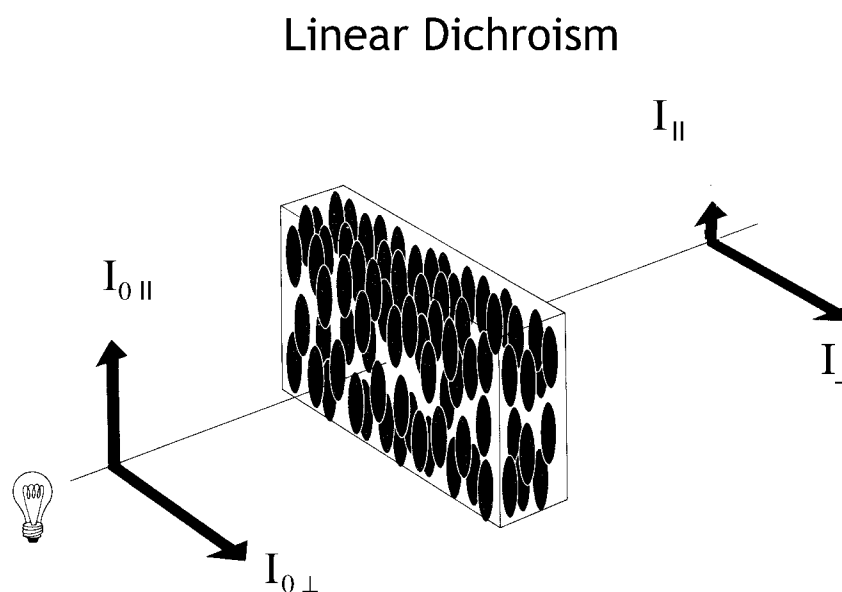


4. Circular Dichroism - Spectroscopy

The optical rotatory dispersion (ORD) and the circular dichroism (CD) are special variations of absorption spectroscopy in the UV and VIS region of the spectrum. The basic principles of the two methods is the interaction of polarized light with optically active substances. If a linearly polarized light wave passes through an optically active substance, the direction of the polarization will change. This change is wavelength dependent. This phenomenon is called *optical rotatory dispersion (ORD)*. Linearly polarized light waves can be described as a superposition of two circularly polarized light waves. If a substance absorbs these two circularly polarized components to a different extent, i.e. if the absorption coefficient for the right circularly polarized component of the polarized light is different from the absorption of the left circularly polarized light, this difference is described as circular dichroism (CD). We will first discuss the physical properties of light that are relevant for the study of circular dichroism of the biological substances.



Schematic diagram of a linear dichroism experiment. Molecules in the sample all are oriented with their long axes in the directions of $I_{\parallel}$.

Polymers oriented in this way are used to generate polarized light in which the electrical field vector oscillates in one direction only.

Figure 4.1. Linear dichroism of oriented molecules.

4.1. Physical concepts.

4.1.1. Linear dichroism and linearly polarized light.

A natural light source is comprised of electromagnetic radiation which is characterized by an isotropic distribution of the electric and magnetic field vectors in space. If the electrical field vector (and the magnetic field vector perpendicular to the electrical field vector) are oscillating only in one direction, then the light is described as *linearly polarized*. The direction of the polarization is given by the electrical field vector.

Linear dichroism of a poly-L-glutamic acid film

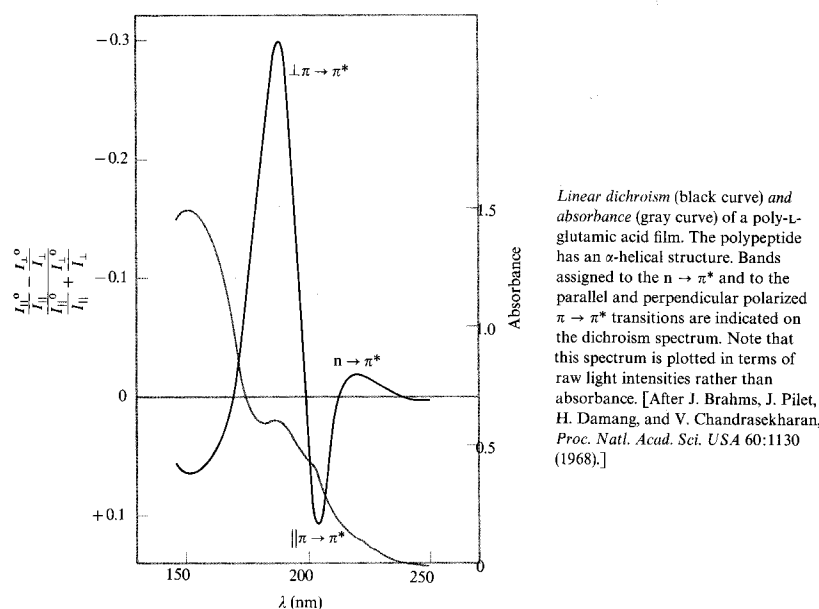


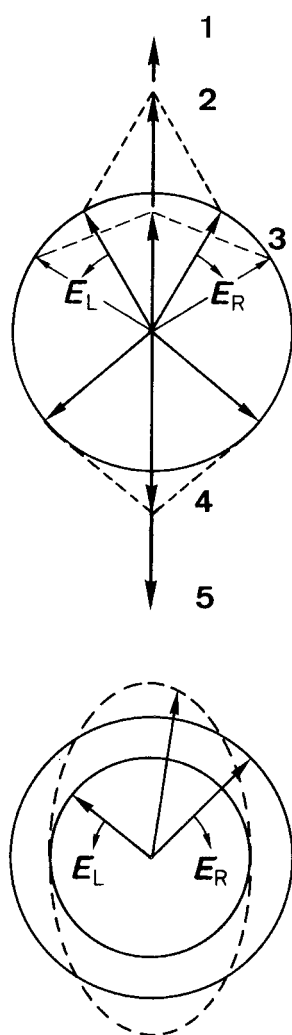
Figure 4.2. Linear dichroism of a poly-L-glutamic acid film. The polypeptide has an α -helical structure. Bands assigned to the $n \rightarrow p^*$ and to the parallel and perpendicular polarized $p \rightarrow p^*$ transitions are indicated on the dichroism spectrum. Note that in this spectrum lines represent the dichroic ratio of reciprocal transmissions rather than the dichroic ratio of absorbances.

Linearly polarized light can be generated using a polarization filter (polarizer). The filter consist of material which is transparent only for one direction. For example a plastic foil, in which the polymer molecules are oriented parallel to each other such that they absorb light oriented parallel to the long axis of the polymer molecules more efficiently than light that is oriented perpendicular to the long axis (see Figure 5.1). The polymer molecules are organized in form of submicroscopic dichroitic crystals that are aligned parallel to each other. Such polarized light can be used to invesitgate the UV absorption spectra of aligned molecules from different directions. Oriented chromophores will absorb polarized light depending on the direction of the electrical field vector of the polarized light and on the direction of the

transition dipole moment (which characterizes the magnitude and the direction by which electronic charge is displaced). Since the concentrations of an oriented substance are often not known, the results of the absorbances of horizontally polarized light and vertically polarized light are given in form of the dichroic ratio:

$$d = \frac{A_{//} - A_{\perp}}{A_{//} + A_{\perp}} = \frac{\epsilon_{//} - \epsilon_{\perp}}{\epsilon_{//} + \epsilon_{\perp}}$$

concentration (c) and pathlength (d) throughout which the light is absorbed is the same for both directions of polarization.



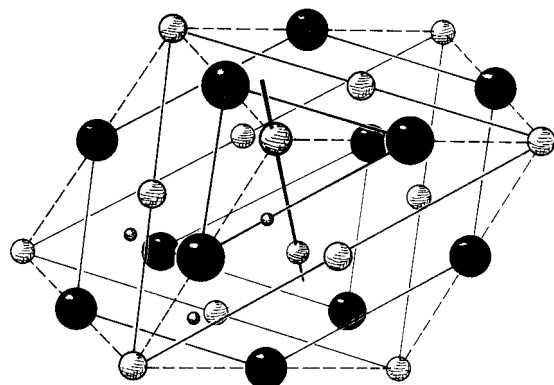
A. Diagram to illustrate the combination of left and right circularly polarized light to linearly polarized light. Both circularly polarized light waves have the same amplitude. The amplitude of the resulting linearly polarized wave oscillates between positions 1 and 5.

B. Elliptically polarized light arises from the superposition of two circularly polarized waves of different amplitudes.

Figure 4.3. Relationship between linearly, circularly, and elliptically polarized light.

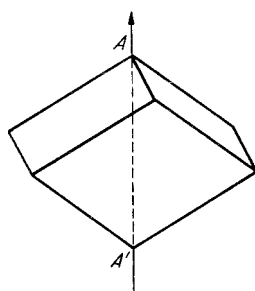
Polarizers other than foils of aligned polymers that are commonly used are based on birefringence, scattering or reflection and make use of the optical anisotropy of the polarizer material. For example, in calcium carbonate crystals (calcite, German "Feldspat") there is a preferred direction along which the crystal has the highest degree of symmetry. This direction is called the crystal's main axes (or

optical axis). The surfaces of the calcium carbonate crystals are parallelograms that are defined by angles of 102° and 78° . The main axes goes through those two corners of the crystal in which the three 102° angles fall together and is symmetrical to the three edges that



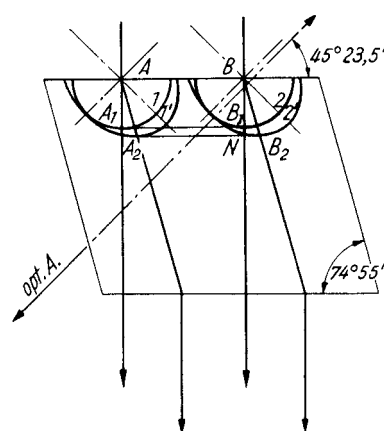
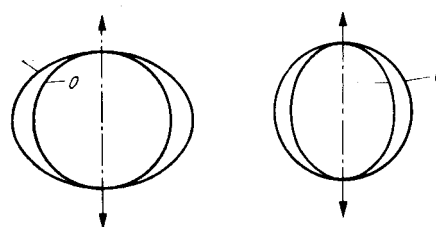
CaCO_3 , ● C, ○ Ca, ○ O.

The calcium carbonate crystal. Oxygen atoms are drawn around one Ca atom. The axis shown is the axis of highest symmetry in the crystal. Symmetry around other directions in space is lower. The axes of highest symmetry is called the *main axis* or *optical axis*.



The areas of calcium carbonate are parallelograms (two angles are 102° and the other two are 78°). The main axis goes through a corner, in which three 102° angles fall together.

Calcium carbonate forms rhombic crystals.



According to the Huygens Principle, light is split into an ordinary and an extraordinary beam when entering a rhombic crystal perpendicular to its surface.

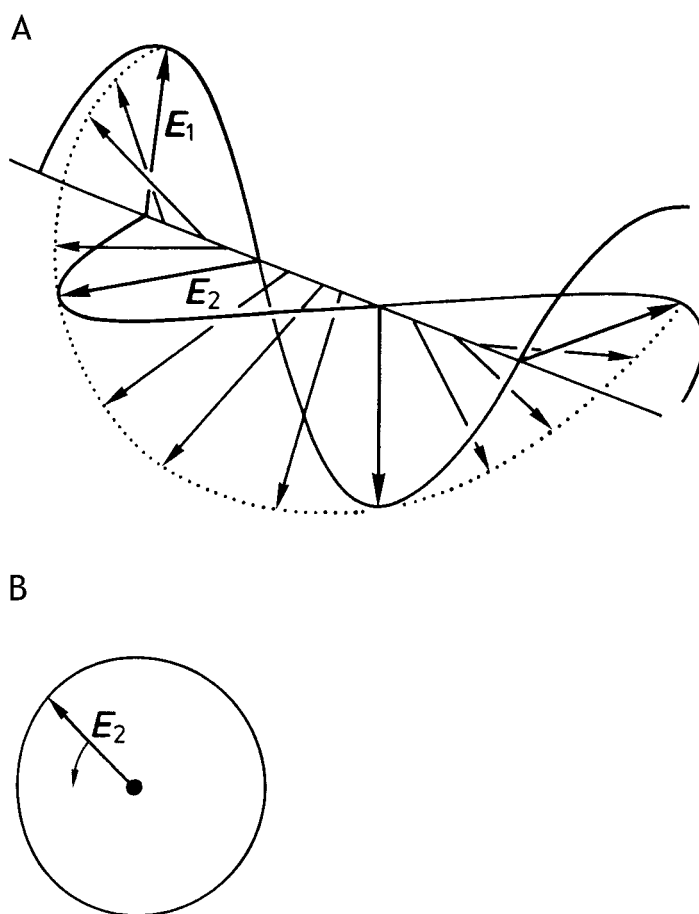
Figure 4.4. Calcite (calcium carbonate) crystal and generation of linearly polarized light by birefringent crystals.

converge in this corner. The speed of light is the same, c_0 , in all directions that are parallel to the crystals main axis (or optical axis), but is dependent on the orientation of the electrical field vector in all other directions. Perpendicular to the optical axis, the speed of polarized light is equal to c_0 , if the magnetic field vector is oriented parallel to the main axis of the crystal (the electrical field vector therefore oriented perpendicular to the main axis). Light that has this direction of polarization is called ordinary light (o-ray). Light of a polarization perpendicular to the ordinary light is called extraordinary light (e-ray). Extraordinary light in

calcite crystals has a speed that is 1.116 times higher than the speed of the ordinary light. If light passes through the calcite crystals at directions in between parallel and perpendicular, the speed of ordinary light is always c_0 , while the speed of the extraordinary light is in between c_0 (c_o) and $1.116 \cdot c_0$ (c_{ao}). According to the Huygens principle (Every point on a primary wavefront serves as the source of spherical secondary wavelets such that the primary wavefront at some later time is the envelope of these wavelets. Moreover, the wavelets advance with a speed and frequency equal to that of the primary wave at each point in space), the wavelets that are originating from the ordinary light travel with the same speed in all the direction, they lie on circles around the point of entry into the crystal while wavelets that originate from the extraordinary light lay on an ellipse. If unpolarized light enters a calcite crystal from a direction perpendicular to the crystals surface, the extraordinary beam is therefore diffracted and the two beams leave the crystal with a parallel displacement. In a Nicol Prism a calcite crystal is sliced diagonally against the natural splitting area. The slices are fixed together with a suitable glue that has a diffraction index slightly smaller than calcite for the ordinary beam. The light beam is entering the glue at an angle larger than the angle of total reflection and is therefore reflected. Since diffraction for the extraordinary beam is smaller, the e-beam will enter the glue at a smaller angle. If the glue is chosen such that its diffraction index is almost equal to the diffraction index of the calcite, it will pass the Nicol prism nearly without any loss of intensity. Therefore the light that exits the Nicol prism will be linearly polarized.

4.1.2. Circularly polarized light

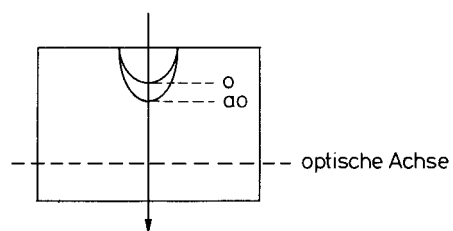
Circularly polarized light can be obtained by superimposing two linearly polarized light beams that have a phase shift of $\lambda/2$ and that have electrical field vectors that are perpendicular to each other. The resultant amplitude vector of the electrical field will then rotate on a spiral perpendicular to the direction of the propagating light wave (see Figure 5.3 below). The displacement of 90° (or $\lambda/4$) is generated by a $1/4$ wave plate that is sliced parallel to the optical axis. A light beam entering the plate perpendicular to the optical axis will not be refracted, but the velocity of the ordinary and the extraordinary light beam will still be different. When exciting the plate, the ordinary and extraordinary light beams will have a phase difference that will depend on the thickness of the plate. The thickness can be chosen such that the phase difference is $\lambda/4$ and the plate is then called a $1/4$ wave plate. If linearly polarized light with an electrical field vector directed at 45° between the fast and the slow axis, is passed through the plate, the field vectors of the ordinary and extraordinary components of this light are perpendicular to each other and the light that exits the plate with a $\lambda/4$ phase shift between the ordinary and extraordinary component is then right circularly polarized. If the angle between the fast and the slow axis is -45° , 315° (i.e. the axis are exchanged) the light is left circularly polarized.



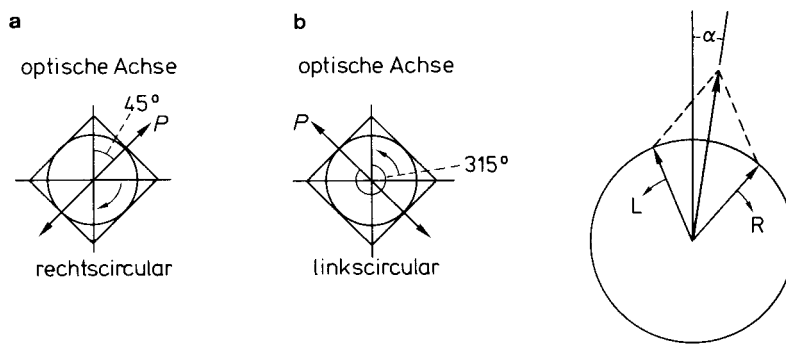
A. Left circularly polarized light can be obtained from a superposition of linearly polarized light. The electrical field vectors of the light waves are perpendicular to each other and the two waves have a phase difference of $+\lambda/4$ with E_1 preceding E_2 . If E_2 would precede E_1 , the resulting wave would be right circularly polarized.

B. Circularly polarized light projected to the direction of proliferation.

Figure 4.5. Generation of circularly polarized light by superposition of two linearly polarized light beams with a phase difference of a quarter wavelength.



Propagation of wavelets of the ordinary and extraordinary ray in birefringent material at perpendicular entrance of the incident light into calcit that is sliced parallel to the optical axis.



Effect of a $1/4$ wave plate. **a** Right circularly polarized light emerges at an angle of 45° between the optical axis and the plane of polarization of the linearly polarized light. **b** at 315° (-45°) the fast and slow axis are exchanged against each other and left circularly polarized light emerges.

Figure 4.6. Effect of a quarter wave plate. Generation of circularly polarized light.

4.1.3. Optical Rotatory Dispersion.

If linearly polarized light passes through an optically active medium (for example an optically active substance in solution), the plane of polarization, i.e. the plane in which the electrical field vector is oscillating, is rotated by an angle α . The angle is proportional to the thickness and the concentration of the optically active component. The angle is also dependent on the wavelength of the linearly polarized light. The phenomenon is therefore called optical rotatory dispersion.

To explain this observation, one must take into account that the speed of light is different for the left and right circularly polarized light in an optically active substance. The ratio of the between the speed of light in vacuum c_0 and in matter c is the refractive index n :

$$n = \frac{c_0}{c}$$

If linearly polarized light passes through a thickness d of an optically active substance, one circularly polarized component will be slower than the other. When the superposition of the both components is monitored after the beam emerges from the sample, is detected, one observes that the direction of polarization is rotated against that of the incident light. For all substances that exhibit such an asymmetrical interaction with light the angle of rotation is given by:

$$\alpha_{(\lambda)} = \frac{180^\circ \cdot d}{\lambda} (n_L - n_R) , \text{ (Grad)}$$

where n_L and n_R are the refractive indices of the left and right circularly polarized components of the light. The specific rotation is given by division of this angle by the concentration and thickness of the sample:

$$[\alpha]_{\lambda} = \frac{\alpha_{\lambda}}{c \cdot d} \quad \left(\frac{\text{Grad} \cdot \text{cm}^2}{\text{mol}} \right)$$

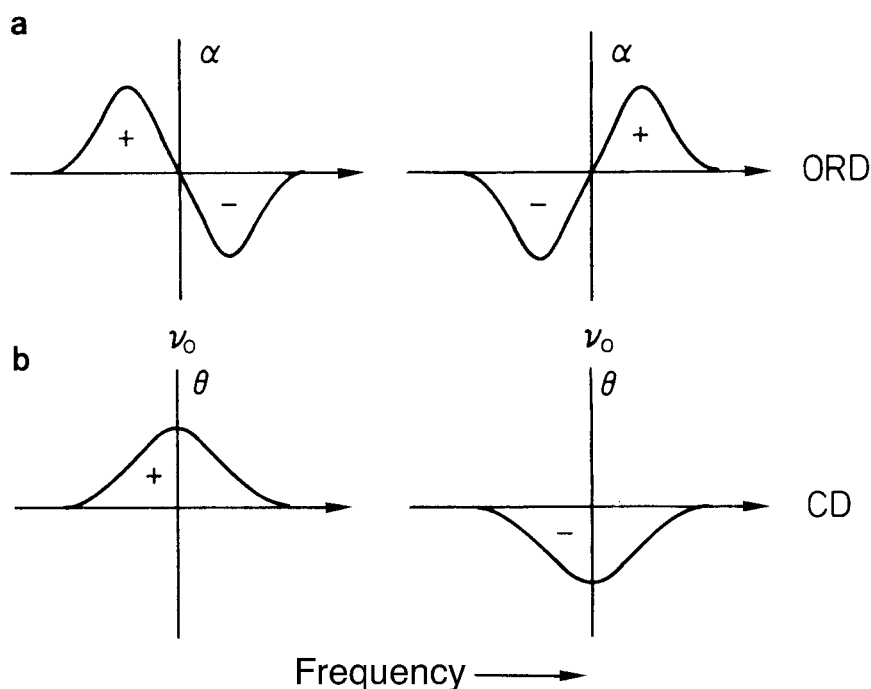
The concentration is calculated in g cm^{-3} and the path length is expressed in cm. With respect to the molar mass M_r in g mol^{-1} the molar rotation:

$$[M]_{\lambda} = \frac{\alpha_{(\lambda)} \cdot M_r}{10 \cdot d \cdot c} \quad \left(\frac{\text{Grad} \cdot \text{cm}^2}{\text{mol}} \right)$$

In applications where optically active polymers are investigated, the molar rotation is normalized by the degree of polymerization, i.e. divided by the number of monomers in the polymer.

Typical molar rotations of amino acids are in the range between 10^5 grad $\text{cm}^2 \text{mol}^{-1}$, which means that a 0.1 mM solution of the optically active amino acids will give a rotation of 1 degree of the polarized light, if the thickness of the cuvette is 1 cm. As a consequence, one can calculate that the difference of the refractive indices of the ordinary and extraordinary components of the light is extremely small ($\Delta n = 1.6 \times 10^{-6}$).

The optical rotation can be positive (right rotating) or negative (left rotating). Direction and angle depend on the wavelength. A chromophore with a positive Cotton effect causes a right rotation at low frequency and a left rotation with higher frequencies. A chromophore with a negative Cotton effect causes a left rotation at low frequencies and a right rotation at the higher frequencies.



Positive and negative Cotton effect of an absorption band. a in the ORD spectrum (+ = rotation to the right), b in the CD spectrum.
(ORD, optical rotatory dispersion)

Figure 4.7. Positive and negative Cotton effect in ORD and CD.

4.1.4. Circular dichroism.

If linearly polarized light passes through an optically active substance, it is possible that not only the speeds of the two circularly polarized components are different, but also the absorption coefficients, ϵ_L and ϵ_R . The difference in the absorption coefficient is determined. Since the absorptions of the left circularly polarized light and the right circularly polarized light are different, elliptically polarized light emerges from the sample. In practice of CD spectroscopy the ellipticity is determined from the difference of the absorption coefficients:

$$\Theta_\lambda = \text{const} \cdot (\epsilon_L - \epsilon_R) \cdot c \cdot d \quad (\text{Grad})$$

where d is the thickness and c the concentration of the sample. Const is given by

$$\text{const} = \frac{180}{4\pi} \ln(10) \approx 33$$

The molar ellipticity is then given by

$$[\theta]_\lambda = \frac{M_r \Theta_\lambda}{10 \cdot d \cdot c} \quad \left(\frac{\text{Grad} \cdot \text{cm}^2}{\text{mol}} \right)$$

M_r is the molar mass in $\text{g} \cdot \text{mol}^{-1}$. If the molar extinction coefficients of the left and right circularly polarized light are known, the molar ellipticity can be expressed as

$$[\theta_\lambda] = 3300 \cdot \Delta\epsilon$$

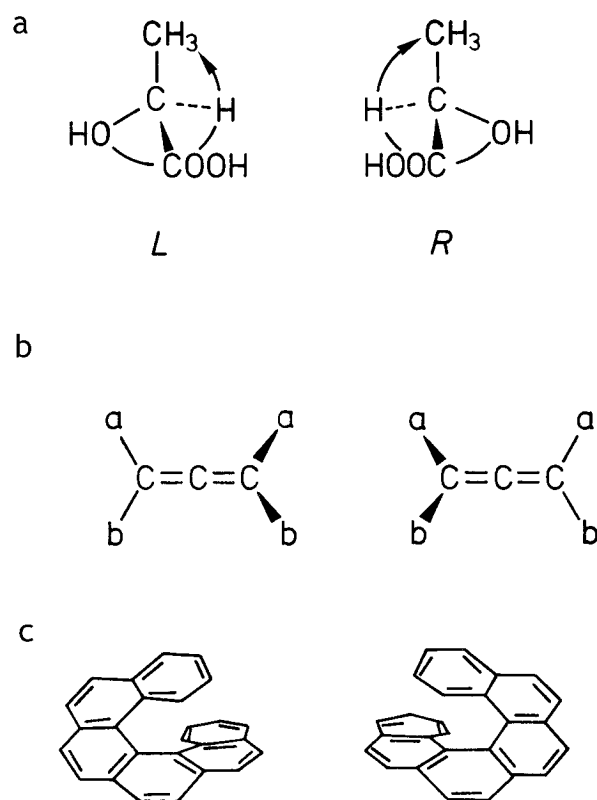
The dependence of the ellipticity on the wavelength of the incident light defines the CD-spectrum. The CD spectrum has the form of an absorption band and a positive and a negative circular dichroism is observed as a function of the wavelength, depending which of the two circularly polarized components is absorbed stronger.

4.2. Optically active chromophors.

In 1848 Louis Pasteur observed that sodium ammonium tartrate from wine crystallizes in two forms that can be separated and that are optically active and rotate polarized light with the same angle into opposite directions (left and right). Such optically active substances are biochemically characterized as D- and L- enantiomers. The two compounds structurally

resemble each other but they lack a symmetry plane. In essence, these substances are like a left and a right hand and cannot be superimposed in space. The compounds are termed chiral. Optical activity is coupled to chirality. Only chiral substances can be optically active.

Most typical examples of chiral substances are compounds that have an asymmetric carbon atom. An asymmetric carbon atom is a carbon atom with four different ligands (see Figure below). An asymmetric carbon atom is often but not always the reason why a substance can be optically active. In quartz crystals, for example, the crystal structure is chiral and causes optical activity. Inorganic substances chirality can also be observed with the presence of an asymmetric carbon atom. Substituted allenes are chiral because of the perpendicular orientation of the ligands. Another example for optical activity without the presence of an asymmetric carbon atom is hexa helicene, which has a spiral like structure arising from the steric repulsion

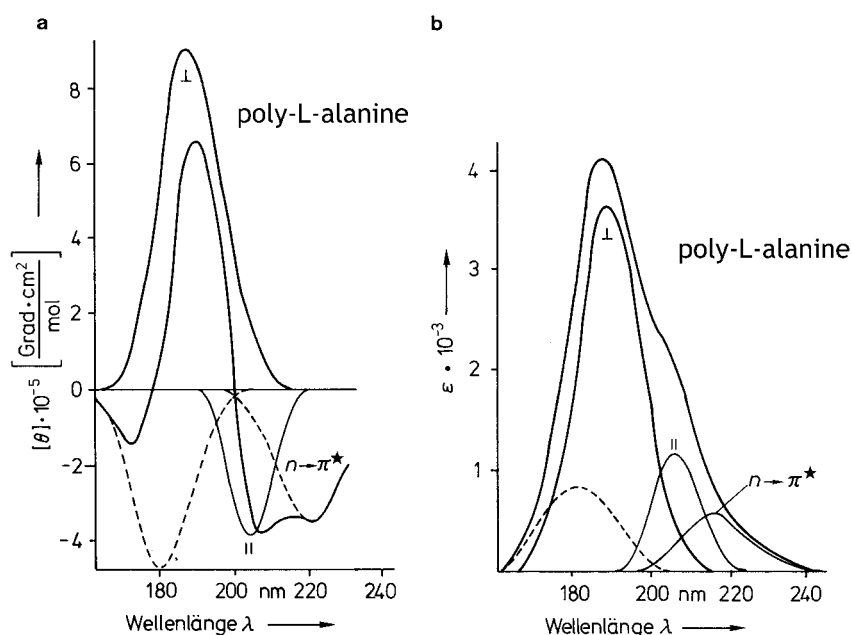


Examples of optically active molecules. a molecules with an asymmetric C atom (here in lactic acid). b substituted allenes. c hexahelicene

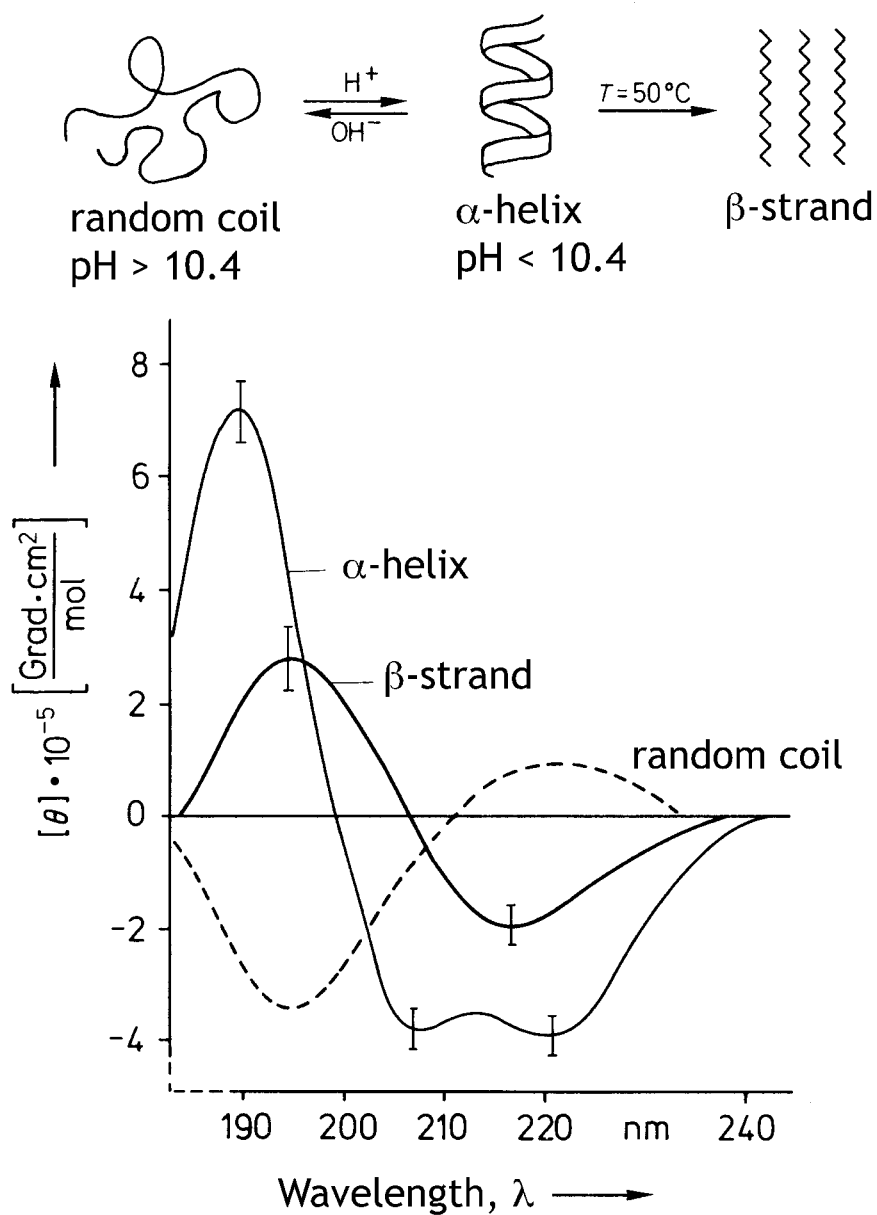
Figure 4.8. Examples of chiral organic molecules.

of hydrogen atoms. In spectroscopy it is not the configuration of a molecule in space that is important for optical activity, but the corresponding asymmetry of the electron distributions.

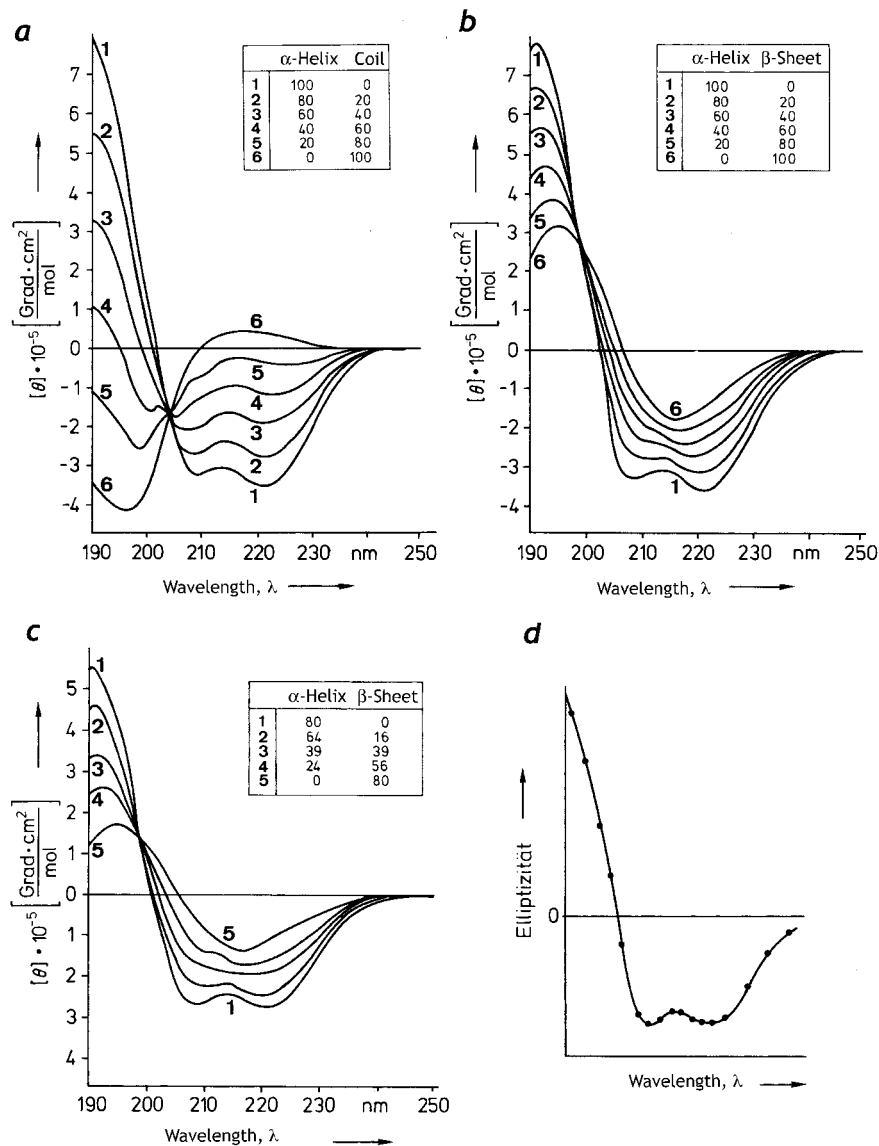
The condition to show optical activity is the possibility that incident light can generate a helical displacement of charges. This displacement explains why there is a preference for the absorption of one of the circularly polarized waves that has a rotatory direction that corresponds to the rotatory direction of possible charge displacement. Another example is 2-Methyl cyclohexanone. While hexanone is optically inactive, the introduction of the methyl group at carbon atom 2 results in an optically active substance. Similarly, the carbonyl chromophore in a peptide is of particular importance. Because of the adjacent asymmetric carbon atom, asymmetry is induced for the carbonyl electrons, resulting in optical activity. In proteins the secondary structure often gives rise to an optically active chromophore by the coupling of oscillators without a center of symmetry or without a symmetry plane. Chirality therefore can result from the overall structure of a macromolecule. This is the basis for the investigation of protein conformation by CD spectroscopy.



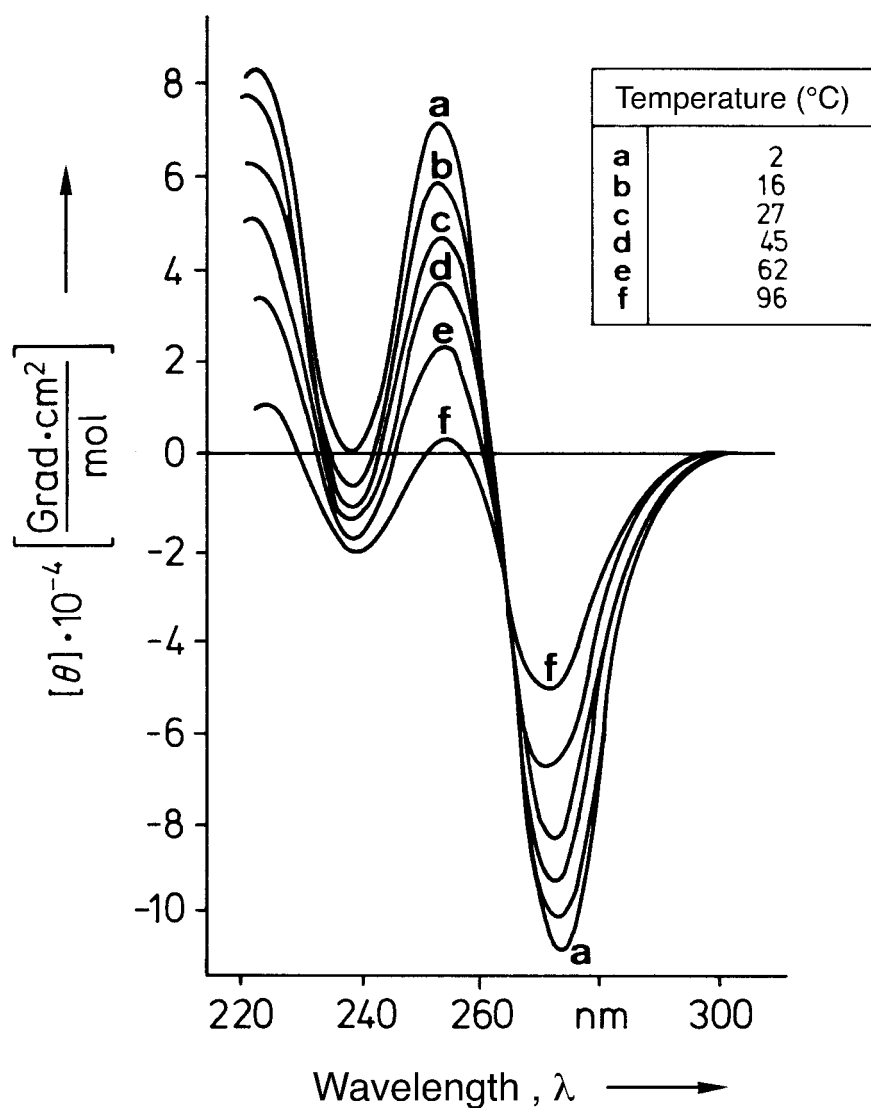
CD spectrum (a) and absorption spectrum (b) of poly-L-alanine in α -helical structure. Bands are assigned to $n \rightarrow \pi^*$ transition at 220 nm and to the parallel and perpendicularly polarized $\pi \rightarrow \pi^*$ transitions at 204 and 190 nm. (After Quadrifoglio, F., Urry, D. W. (1968), J. Ann. Chem. Soc. 90, 2755)



Conformational changes and CD-spectra of poly-L-lysine in aqueous solution. (After Greenfield, N., Fasman, G. (1969), *Biochemistry* 8, 4108)



Calculated CD spectra with different content of α -helix, β -sheet, and random coil. The percentages of the individual secondary structure elements of the depicted spectra are given in the corresponding table. **a** variable content of α -helix and random coil, **b** variable content of α -helix and β -sheet, **c** variable content of α -helix and β -sheet at 20% random coil, **d** CD spectrum of myoglobin: the filled circles were calculated from spectra obtained from the different conformations of poly-L-lysine and gave a composition of 68.3% α -helix, 4.7% β -sheet, and 27% random coil. (After Greenfield and Fasman, 1969, *Biochemistry* 8, 4108).



Temperature dependence of the CD-spectrum of Adenosine-5'-mononucleotide in 2 M NaCl solution. At low temperatures, the bases are stacked upon each other, at high temperatures, bases are dissociated. (After Miles, D. W., Urry, D. W., 1967, J. Phys. Chem. 71, p4448).