Circular Dichroism & Optical Rotatory Dispersion

Many biomolecules are $\alpha$-helical!
How can we measure the amount and changes in amount of helical structure of a biomolecule in solution?

Can we use x-rays?

[Diagram: Need a crystal!]

Can we use radiowaves (i.e. NMR)?

[Diagram: Can't measure changes very readily]

Can we use polarized light?
Plane polarized light

Circularly polarized light

Take two polarized waves: one along x and the other along y, but with a phase difference of $\lambda/4$

\[ E_x = E_0 \sin\left(\frac{2\pi z}{\lambda}\right) \]

\[ E_y = E_0 \cos\left(\frac{2\pi z}{\lambda}\right) \]
clockwise or left-handed

from http://www.bip.bham.ac.uk/osmart/bcm201_cd/cd_movie/index.html
- Right-handed or anticlockwise circularly polarized light is obtained by using
  \[ E_y = E_0 \cos\left(\frac{2\pi z}{\lambda}\right) \]

- Combining left and right circularly polarized waves of equal amplitudes results in polarized light.

- Combining left and right circularly polarized waves of unequal amplitudes results in elliptically polarized light.

http://www-structure.llnl.gov/cd/cdtutorial.htm
Circular dichroism

We pass either left-handed or right-handed light through an “optically active” substance.

What we find is that the amount of light absorbed by the substance depends on whether the light is left-handed or right-handed circularly polarized.

Circular dichroism, at a given wavelength $\lambda$, is defined as:

$$\Delta \varepsilon = \varepsilon_L - \varepsilon_R$$

where $\varepsilon$ is the extinction coefficient. A molecule is “optically active” if it is chiral or asymmetric.
An absorption band can be characterized by its rotational strength, which is given by

\[ R = \frac{(2.303)(3000)hc}{32\pi^3N_A} \int \frac{\Delta \varepsilon}{\lambda} d\lambda \]

where \( h \) is Planck’s constant and \( c \) is the speed of light.

Since the left- and right-handed light will be absorbed differently, we will have ellipticity. The occurrence of ellipticity is called circular dichroism.
Circular dichroism is observed only for wavelengths where the substance absorbs light. For all other wavelengths, we will have optical rotation.

Optical rotation occurs as a consequence of a different refractive index for left and right circularly polarized light components.
Optical rotation or circular birefringence results from different indices of refraction for right- and left-circularly polarized light. The angle of rotation depends on the nature of the substance, the thickness of the sample d, and the concentration C, such that

$$\alpha = [\alpha] \, dC$$

where [$\alpha$] is the specific rotation. We can measure a change in the optical rotation with wavelength. This is known as optical rotatory dispersion (ORD).
These hypothetical CD and ORD spectra illustrate a number of things:

1) Cotton effect – which is a change in sign in the sign of rotation.

How does this arise? ORD is analogous to refractive index so let’s take another look at refractive index.
What we need to determine now is how this induced dipole oscillation is affected by changes in wavelength. To do this, let us perform the following thought experiment:

- Record rotates slower than the ladybird (long wavelength)
- Record rotates at the same speed as the speed of the ladybird (resonance)
- Record rotates faster than the ladybird (short wavelength)
The same holds of the optical rotation or refractive index.

2) The quantity used to describe optical rotatory dispersion is not the specific rotation $[\alpha]$ but rather the molar rotation $[m']$:

$$[m'] = [\alpha] \frac{3}{n^2 + 2} \frac{M}{100}$$

where $n$ is the refractive index of the medium and $M$ is the molecular weight of the solute. The term $(3/n^2 + 2)$ is a correction factor which accounts for the slight polarizability of the medium and its effect on the solute.
The previous equation relates the molar rotation to the specific optical rotation. What is also needed is the relationship between \([m']\) and wavelength:

\[
[m'] = \frac{96\pi N_A}{hc} \frac{R \lambda_0^2}{\lambda^2 - \lambda_0^2}
\]

where \(R\) is the rotational strength, \(h\) is Planck’s constant, \(c\) is the speed of light, and \(\lambda_0\) is the wavelength of the band. This equation is the Drude equation and describes the response of an undamped harmonic oscillator driven by a periodic force.

Putting \(\lambda = \lambda_0\), we see that this equation predicts that \([m'] \to \infty\). This is not what is observed experimentally. To get the correct description, a damping term must be included.
3) CD and ORD are related since both rely on the same underlying phenomenon. They are related by the Kronig-Kramer relations. For example,

\[ [m'] = (2.303) \frac{9000}{\pi^2} \int_0^\infty \Delta \varepsilon_{\lambda'} \frac{\lambda'^2}{\lambda^2 - \lambda'^2} \, d\lambda' \]

So why have techniques been developed to measure both CD and ORD?
- Circular dichroism is an absorptive quantity and ORD is dispersive. CD is a higher resolution method since we measure (relatively narrow) absorption bands. In ORD, the dispersive peak is quite spread out – if two bands are close to each other then it is difficult to distinguish them.

- ORD measurements can be performed at wavelengths where the substance being investigated does not necessarily absorb light.
We have introduced the concept that circularly polarized light is absorbed differently if it is left- or right-handed (CD) or that light is refracted differently through a sample depending on whether it is left- or right-handed (ORD). But why should this be? What is it about the substances that results in this effect?

\[ \alpha = 3640^\circ \]

Coronene

Hexahelicene

\[ [\alpha] = 3640^\circ \]
- The electric field will induce an oscillating dipole $\mu$ which will have a component along the helix axis (recall the example on benzene shown earlier).
- Electric field is always accompanied by a magnetic field.
- An oscillating magnetic field parallel m to the helix axis will induce current in the helix.
The magnetic field for circularly polarized light has a component which is parallel to the electric field. This is 90 degrees out of phase with the electric field parallel to the helix axis. But its derivative will be in phase.

Therefore both the electric field and the magnetic field will contribute to an electron displacement along a helical path. So we will have an electric and a magnetic dipole moment.

Now if the circularly polarized light is right-handed, the phase of the light and that of the electron are the same. In other words, both the electric field and the magnetic field act in concert on the electron.

If the circularly polarized light is left-handed, then there is a phase difference of 180 degrees. In this case, the electric and magnetic fields acting on the electron are in opposite directions.
A consequence of this is that for a left-handed helix, the same arguments would apply – but to the opposite handedness, i.e. left-handed circularly polarized light would result in both the electric field and magnetic field acting in concert on the electron.

Therefore a left-handed helix should display optical rotatory effects which are just the opposite to those given by a right-handed helix.

One enantiomer of hexahelicene is optically active. What about a racemic mixture?
Previously we defined $R$, which is a rotational strength or optical rotatory power. It can be shown (quantum mechanically) that $R$ is

$$R = \text{Im} \ (\mu \cdot m)$$

i.e. the imaginary component of the scalar product between the electric and magnetic moments. For most purposes, we can say that

$$R = \mu m \cos \theta$$

where $\theta$ is the angle between the two dipoles.
The electric dipole moment and the magnetic dipole moment (coming out of the plane of the page) are perpendicular.

As long as there is a plane of symmetry in the molecule, it remains optically inactive.

Types of asymmetry in macromolecules which lead to optical activity:

1) at the primary structure level – C\textsuperscript{\alpha} carbons are chiral

2) at the secondary structure level – many biopolymers are helical
CD signals for GCN4-p1


Figure 3: 34 μM GCN4-p1 in 0.15 M NaCl, 10 mM phosphate pH 7.0

- main application of CD
3) at the tertiary structure level – e.g. asymmetries in binding pockets

free tyrosine
only weakly optically active

tyrosine in a binding pocket
strongly optically active
**Figure 1:** Top: Schematic drawing of the backbone conformation of hirudin (variant 1) N-terminal core domain 1–51 (28) using the program RasMol, v. 2.6 (25), and the PDB file 1HIC. Positions of Thr$_{45}$ and Pro$_{46}$ are indicated by sticks. The three disulfide bonds are symbolized by lines. Bottom: Amino acid sequence of hirudin used in this paper. Merely, the two N-terminal amino acids (Leu-Thr-) differ from the natural sequence of hirudin (variant 1) (Val-Val-). Plain lines and asterisk represent disulfide bridges and the phosphorylated threonine residue, respectively.

**Figure 2:** Far-UV CD spectra of hirudin at pH 6.0 (●) and pH 8.0 (○) and pThr$_{45}$-hirudin at pH 6.0 (■) and pH 8.0 (□). The spectra were obtained in 10 mM sodium formate (pH 4.0), 10 mM MES (pH 5.0, 6.0, 6.5), 10 mM sodium phosphate (pH 7.0, 7.5), 10 mM Tris-HCl (pH 8.0, 9.0), and 10 mM sodium borate (pH 10.0) at 20 °C. The protein concentrations were 0.1 mg/mL. The inset shows a titration curve derived from the ellipticities at 200 nm of far-UV CD spectra of pThr$_{45}$-hirudin at various pHs. The solid line represents the result of a fit according to the Henderson–Hasselbalch relationship (pK$_a$ = 7.2).

**Reference:** Kipping et al., Biochemistry, 40, 7957 (2001).
A few practical aspects

- **Additives, buffers and stabilizing compounds**: Any compound which absorbs in the region of interest (250 - 190 nm) should be avoided. A buffer or detergent or other chemical should not be used unless it can be shown that the compound in question will not mask the protein signal. For instance imidazole cannot be used below 220 nm because it overwhelms the spectrum from then on. Therefore ensure that only the minimum concentration of additives are present in the protein solution.

- **Protein solution**: From the above follows that the protein solution should contain only those chemicals necessary to maintain protein stability, and at the lowest concentrations possible. Avoid any chemical that is unnecessary for protein stability/solubility. The protein itself should be as pure as possible, any additional protein or peptide will contribute to the CD signal.

taken from: http://www-structure.llnl.gov/cd/cdtutorial.htm
- **Contaminants**: Unfolded protein, peptides, particulate matter (scattering particles), anything that adds significant noise (or artifical signal contributions) to the CD spectrum must be avoided. Filtering of the solutions (0.02 um syringe filters) may improve signal to noise ratio.

- **Data collection**: Initial experiments are useful to establish the best conditions for the "real" experiment. Cells of 0.5 mm path length offer a good starting point.

**Typical Initial Concentrations:**

*Protein Concentration*: 0.5 mg/ml  
*Cell Path Length*: 0.5 mm  
*Stabilizers (Metal ions, etc.):* minimum  
*Buffer Concentration*: 5 mM or as low as possible while maintaining protein stability

-may need to change protein concentration to produce the best data. Changing this has a profound effect on the data, so small increments or decrements are called for. If that does not produce reasonably good data, a change in buffer composition may be necessary. If absorption poses a problem, cells with shorter path (0.1 mm) and a correspondingly increased protein concentration and longer scan time can help.