Optical Spectroscopy
Chapter E
Feb 10, 2014

Definitions

- Spectroscopy is the study of the interaction of electromagnetic radiation with matter.
- Absorption- radiation strikes a molecule and causes a shift from ground state to higher energy.
- Emission - going from higher to lower energy

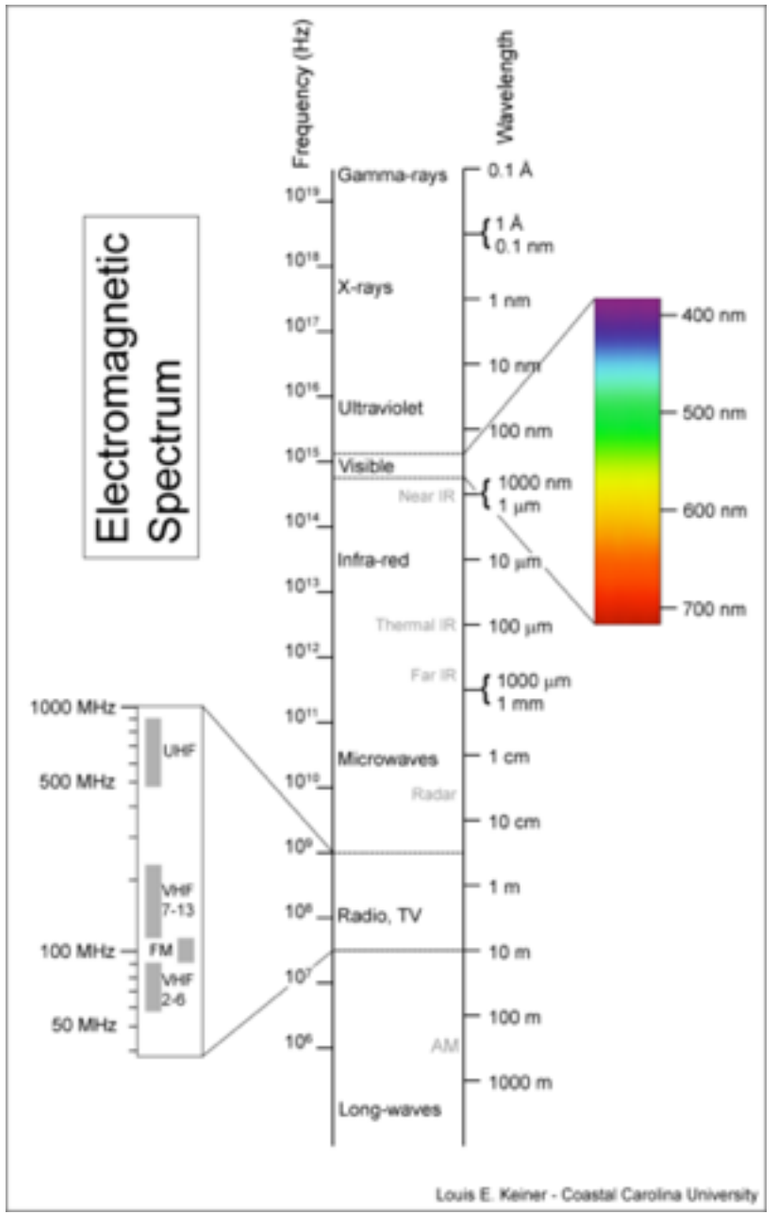
Types of Spectroscopy

- Ultraviolet and Visible spectroscopy monitors changes in electronic state (measure concentration)
- Circular dichroism - absorption of polarized light (secondary structure prediction)
- Fluorescence and phosphorescence are types of emissions
- Infrared
- NMR - nuclear magnetic resonance - the absorption of radiofrequency by nuclei in a magnetic field

Optical Spectroscopy of Biological Samples

- Proteins and nucleic acids have specific absorption signatures in the UV and IR spectral ranges.
- Biological macromolecules are colorless but prosthetic groups, such as retinal in the vision proteins or the heme group in myoglobin, haemoglobin and the cytochromes, which absorb visible light.
- The absorption spectra of these chromophores depend on their local environment and can be used to probe conformational changes and dynamics in the macromolecule.
- Enzyme activity can be measured with great sensitivity through the observation of the specific optical absorption patterns of substrates, products and cofactors.

Electromagnetic Spectrum



- Electromagnetic spectrum in terms of wavelength, frequency and Energy
- $c=\lambda v$
  - c= speed of light in a vacuum $3x10^8$ m/s
  - v= frequency in Hertz (Hz s^{-1})
  - λ= wavelength
- Energy=hv
  - h = Planck constant $6.6261x10^{-34}$Js

Absorption Spectroscopies

Table E1.1. *Absorption spectroscopies*

λ(m)	ν (Hz s^{-1})	E (kJ mole^{-1})	Method
~ 3	$\sim 10^{8}$	$\sim 10^{-4}$	Radio-frequency NMR
~ 0.1–0.01	$\sim 10^{9}$–10^{10}	$\sim 10^{-1}$–10^{-2}	Microwave rotational and EPR spectroscopy
$\sim 10^{-4}$–10^{-5}	$\sim 10^{12}$–10^{13}	~ 1–10	IR vibrational spectroscopy
$\sim 8\times10^{-7}$–4×10^{-7}	$\sim 5\times10^{14}$–10^{15}	~ 200	Visible electronic spectroscopy
$\sim 10^{-7}$	$\sim 4\times10^{15}$	$\sim 10^{3}$	UV electronic spectroscopy
$\sim 10^{-10}$	$\sim 10^{18}$	$\sim 10^{6}$	X-ray absorption electronic spectroscopy
$\sim 10^{-13}$	$\sim 10^{21}$	$\sim 10^{9}$	γ-ray Mössbauer spectroscopy

Absorption - theory

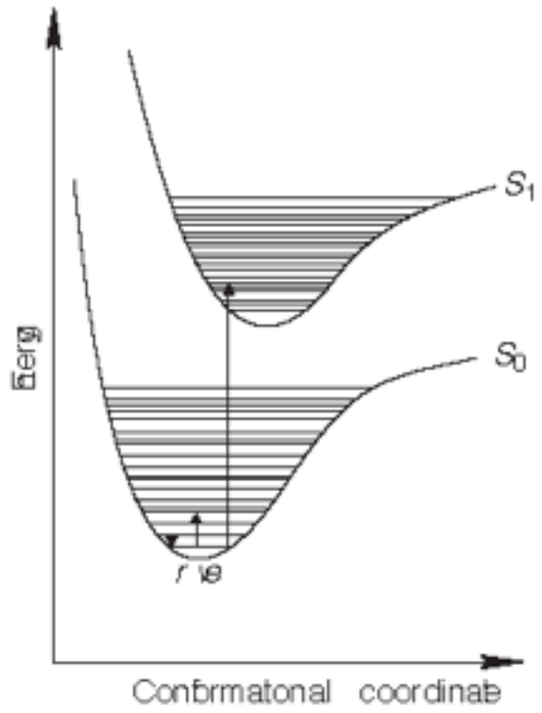


Fig. E1.2 Potential energy diagram of the lowest (S_0) and first excited (S_1) electronic states in a molecule. *r*, *v*, and *e* are rotational, vibrational and electronic transitions, respectively. (After Cantor and Schimmel, 1980.)

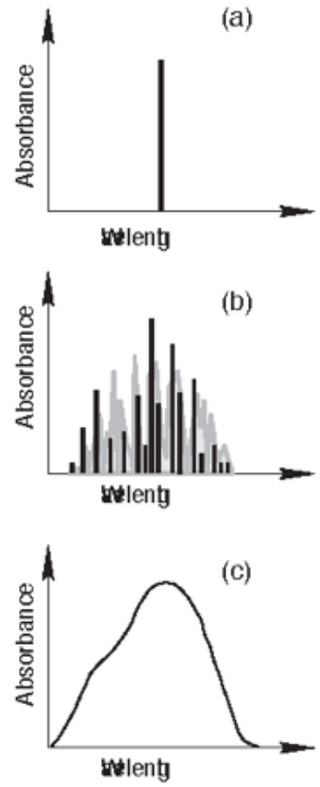


Fig. E1.3 Absorption spectrum of a molecule with one type of electronic transition: (a) theoretical, at very low temperature; (b) theoretical at ambient temperature; (c) observed. (After Cantor and Schimmel, 1980.)

Beer-Lambert Law

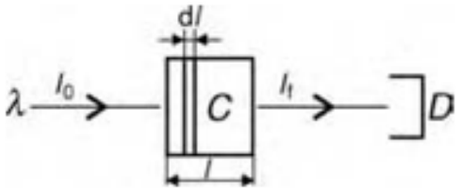


Fig. E1.1 Schematic of an experiment to measure absorbance. A beam of wavelength, λ, and intensity, I_0 is shone on a sample cell of path lenth, l, containing a solution of concentration, C (moles litre^{-1}). The final transmitted intensity, I_f, is measured by the detector, D.

Consider the simple set-up in Fig. E1.1. The intensity of light absorbed, $-\mathrm{d}I$, by the molecules in the thin sample slab of thickness, $\mathrm{d}l$, and unit area is proportional to the number of moles in the slab, $C\mathrm{d}l$, and to the incident intensity, I:

$$-\mathrm{d}I = C\varepsilon'(\lambda)I\mathrm{d}l \tag{E1.1}$$

where ε', the proportionality constant, depends on the wavelength and on the particular molecular type. It is called the *molar extinction coefficient* and has usual units of (mole litre^{-1})$^{-1}$ cm^{-1}, or M^{-1} cm^{-1}.

Integrating Eq. (E1.1) over the sample path length, l, we obtain

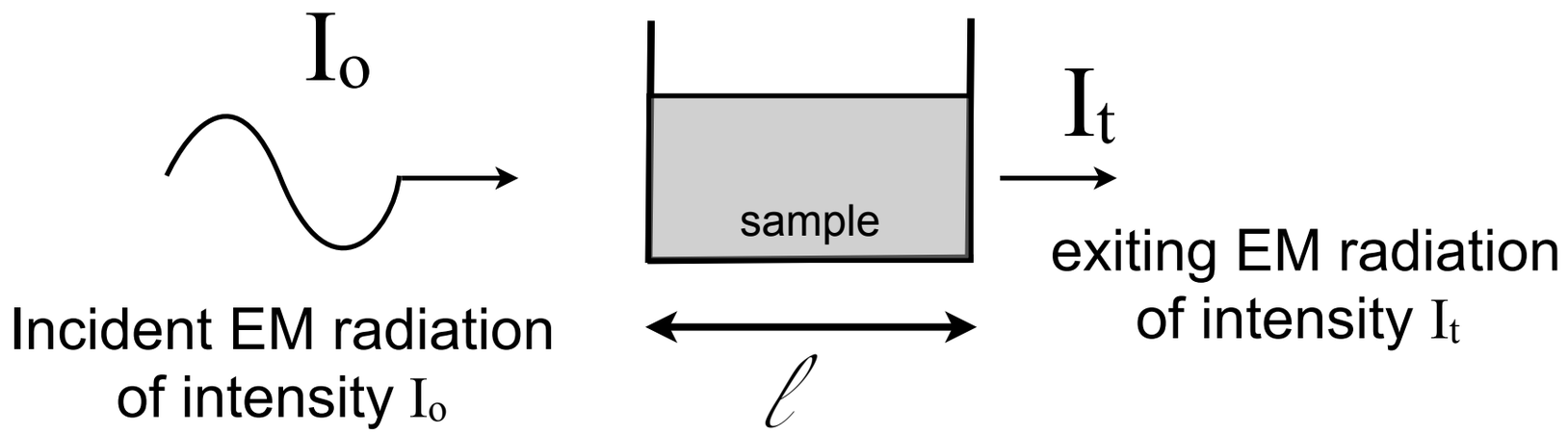
$$\ln(I_0/I_f) = C\varepsilon'(\lambda)l \tag{E1.2}$$

where I_f is the final intensity transmitted by the sample (Fig. E1.1).

Equation (E1.2) is usually expressed in terms of the base 10 logarithm:

$$\log(I_0/I_f) = C\varepsilon(\lambda)l \equiv A(\lambda) \tag{E1.3}$$

Beer-Lambert Law

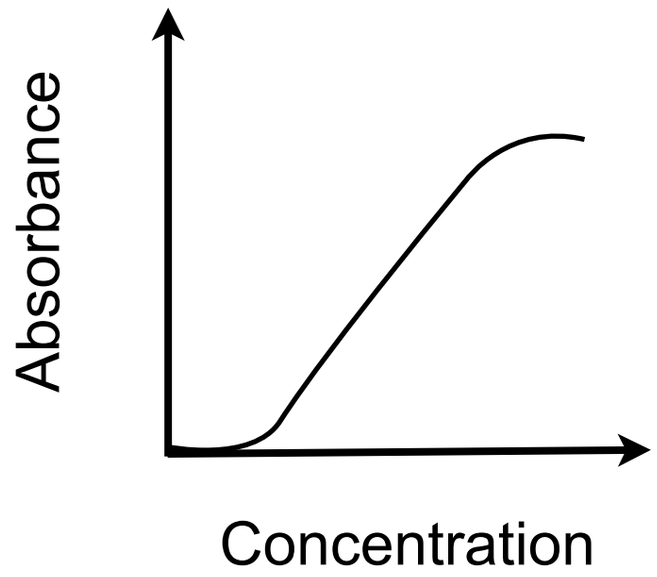


$$A=\log(I_o/I_t)=\varepsilon C\ell$$

- Absorption is A
- ε is the molar absorptivity or molar extinction coefficient. Units M^{-1} cm^{-1}. This is a characteristic of the sample.
- ℓ is the path length. Usually given in cm (1 cm is standard)
- sample contains absorbing substance of concentration c
- What are the important considerations?
- solution does not absorb
- walls of the cell do not absorb at the given wavelength

Breakdown of Beer-Lambert

- Beer-Lambert should be linear but it does not hold at high and low concentrations
- Need to stay within the linear range



- Also light scattering instead of absorption

Spectrometers

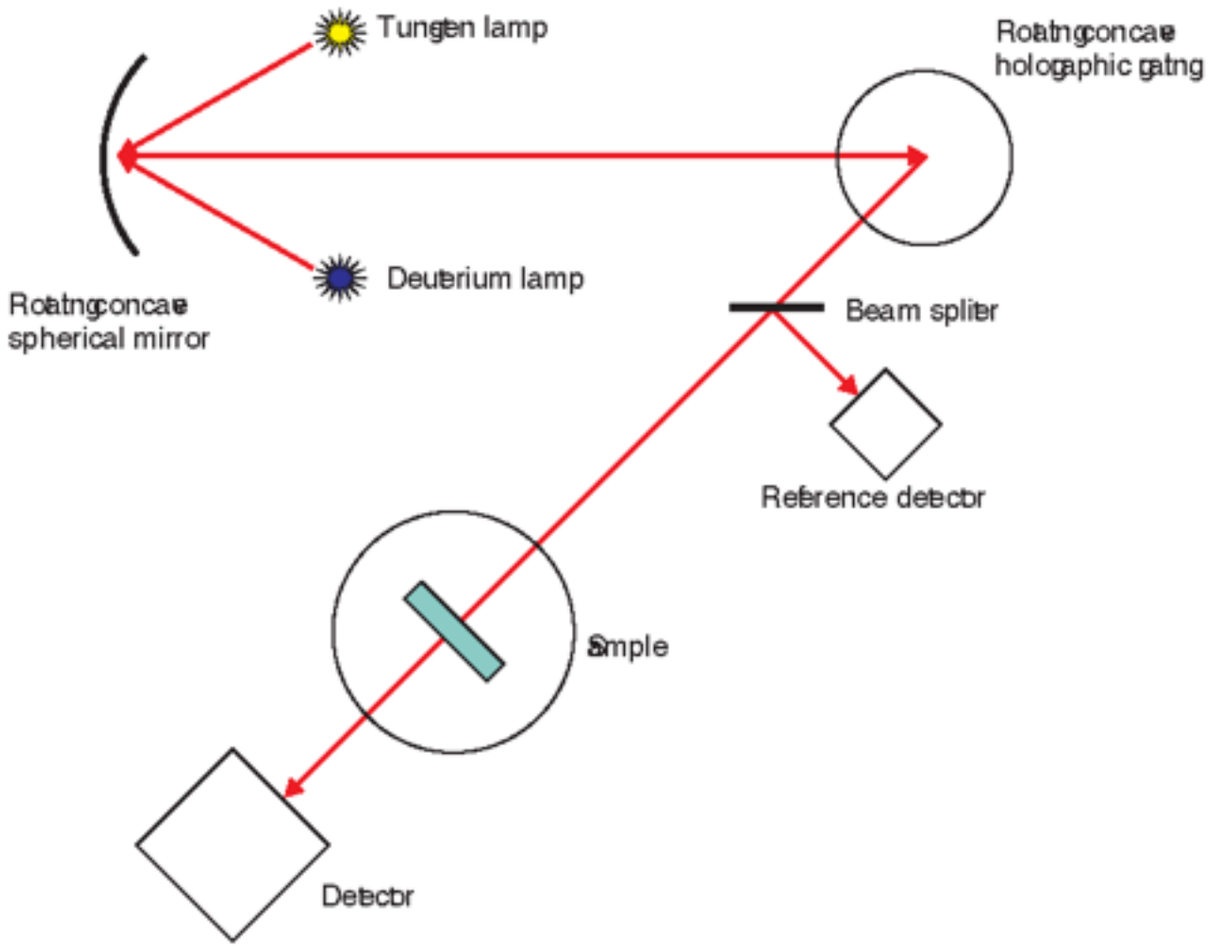


Fig. E1.4 The optical path in a single/split beam spectrophotometer. The tungsten and deuterium lamps provide light in the visible and UV ranges, respectively. The rotating concave holographic grating geometrically spreads out the beam into its wavelength components, so that the entire wavelength range is measured simultaneously by a photodiode array (detector). A beam splitter diverts part of the beam to a reference detector for continuous calibration.

Absorption of Proteins

The absorption of proteins (three ways)

- electronic bands in the peptide group (at 170--220 nm),
- aromatic amino acid side-chains close to 280 nm
- prosthetic groups, cofactors, enzyme substrates or inhibitors

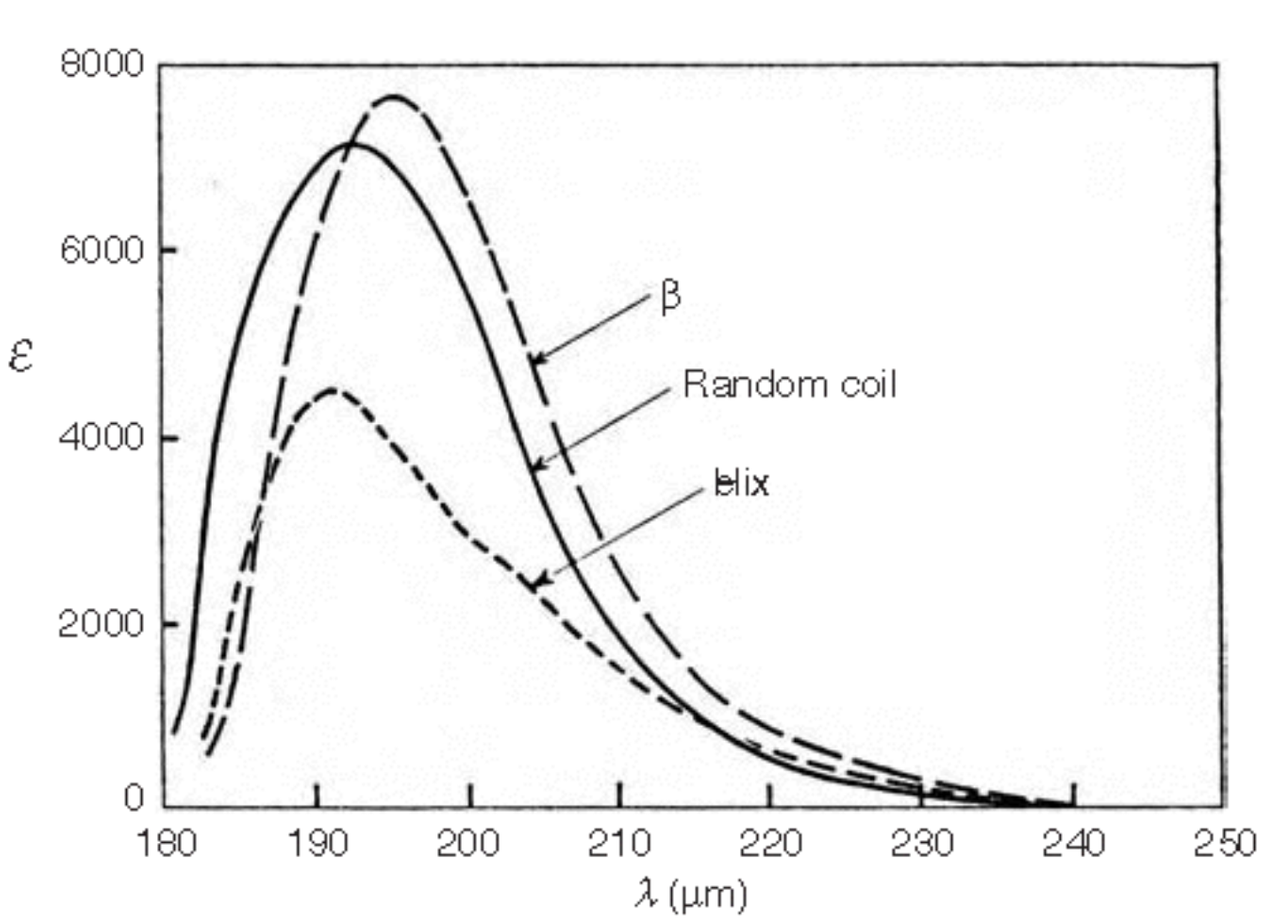
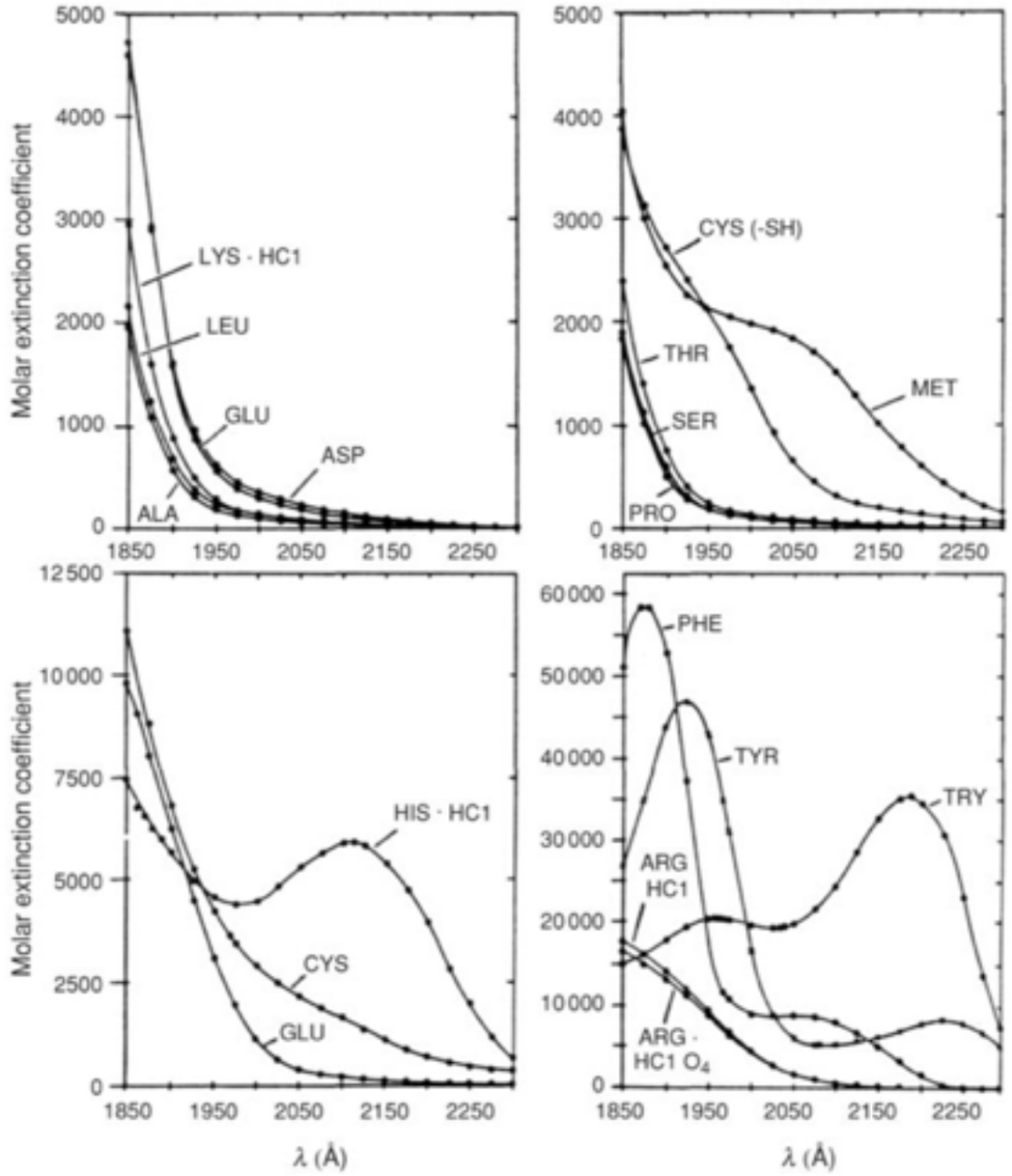


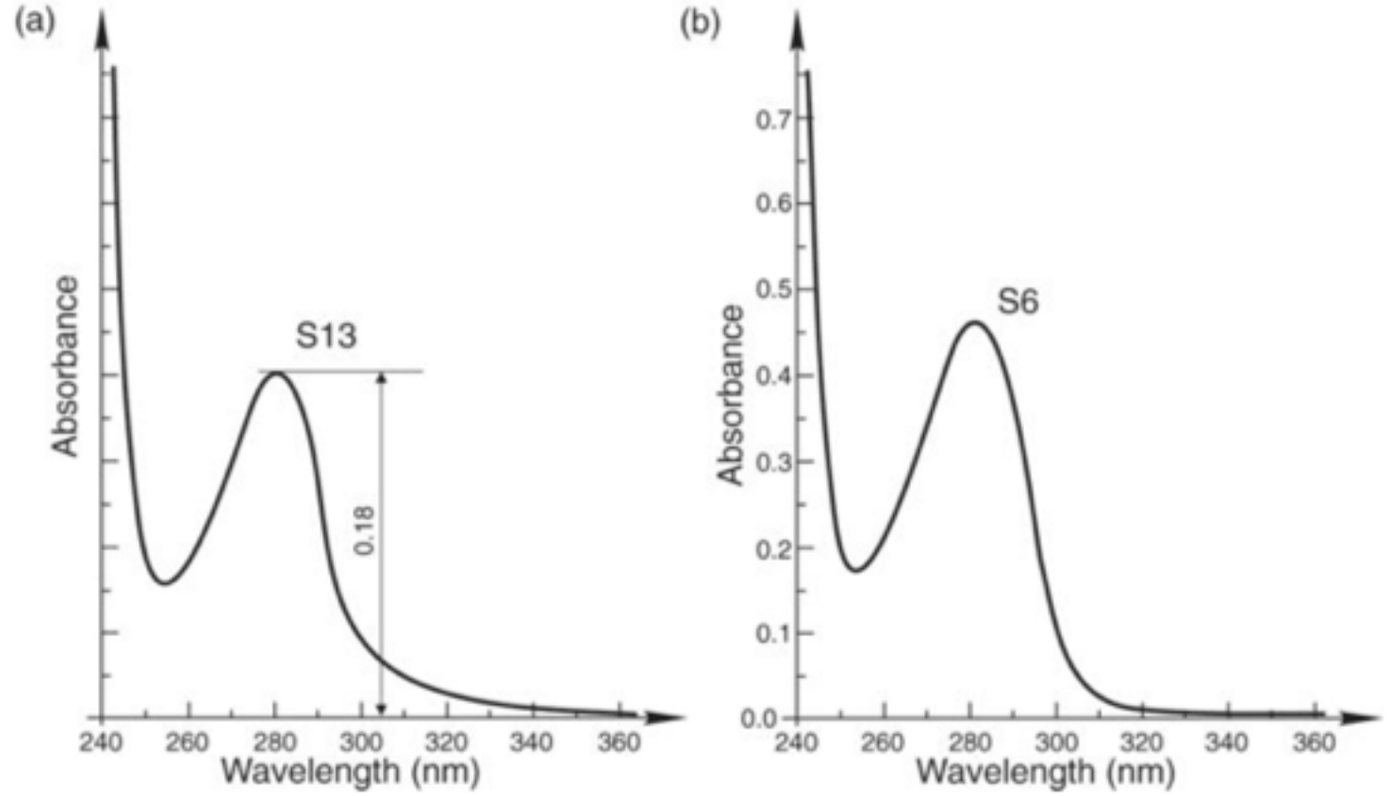
Fig. E1.7 UV absorption spectra of poly-L-lysine hydrochloride in aqueous solution: random coil at pH 6.0, 25 °C; helix at pH 10.8, 25 °C; *β*-form at pH 10.8, 52 °C. The *x*-axis is the wavelength in nano-metres (the 'milli-micron' is an old fashioned unit, now replaced by 'nano-metre'). (From Rosenheck and Doty, 1961, reviewed by Wetlaufer, 1962.)

Absorption Spectra of Amino Acids



- Amino acids tryptophan, tyrosine, cysteine (not shown) and phenylalanine are the only ones that absorb at wavelengths greater than 230nm
- $\pi \rightarrow \pi^*$ transitions of the aromatic ring

Protein concentration Measurements



- Molar extinction coefficients at 280 nm, calculated from amino acid compositions (in particular, Tyr, Trp and Cys contents)

- unfolded proteins in guanidine hydrochloride,
- native proteins may differ considerably. Why??.
- Best to measure absorption over a range of wavelengths
- What is contributing to the absorption at 320nm on the left panel?

Quantification of Proteins

- Measuring protein concentration using absorbance at 280nm
  - Advantage - Nondestructive
  - Disadvantage - depends on the number of Tryptophan, Tyrosine and cysteine
- Estimate ε_{280} (M^{-1} cm^{-1})=(#W)(5,500) + (#Y)(1,490) + (#C)(125)
  - Dependent on knowing the protein sequence
- [protein] (mg/ml) = (1.55 x A_{280}) – (0.76 x A_{260})
  - Estimation if protein sequence is unknown.
- Measure at the peptide bond (200-220nm)
  - Advantage - more sensitive, not dependent on amino acid composition
  - Disadvantage - buffer effects, some amino acids absorb in this range, influences of secondary structure

Effect of protein-associated groups

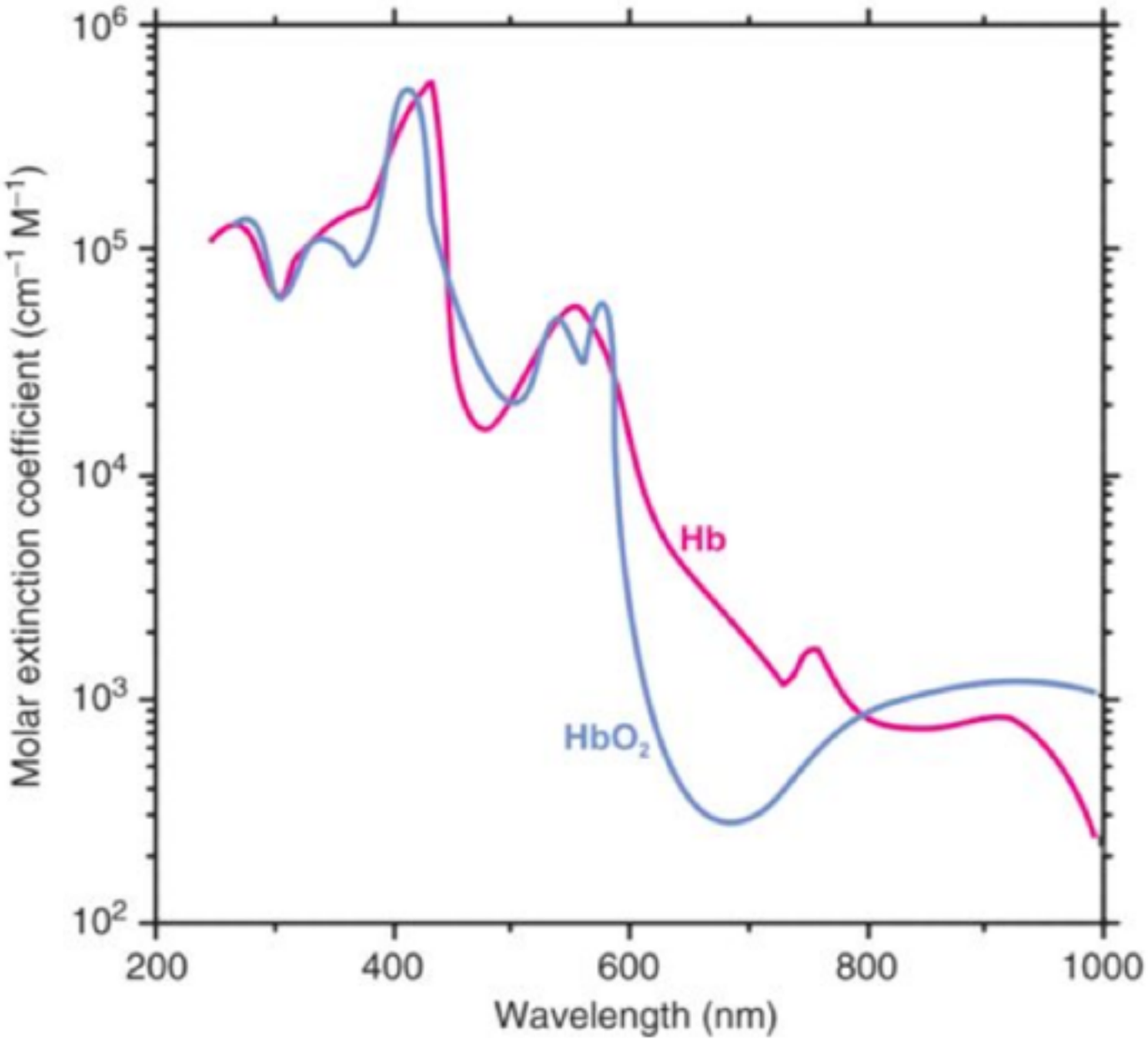
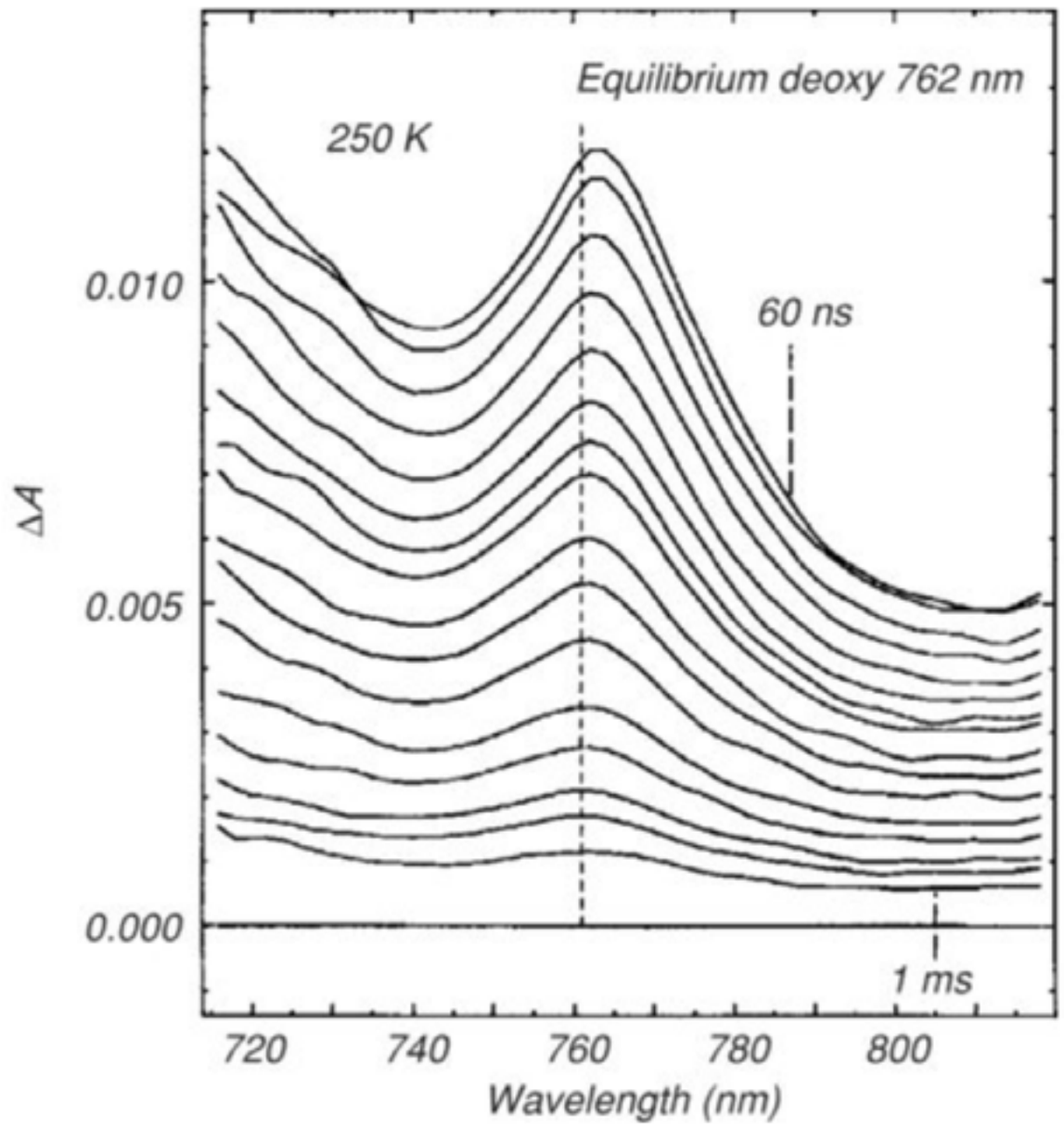


Fig. E1.12. Absorption spectra of haemoglobin and oxygen-bound haemoglobin.

Kinetics



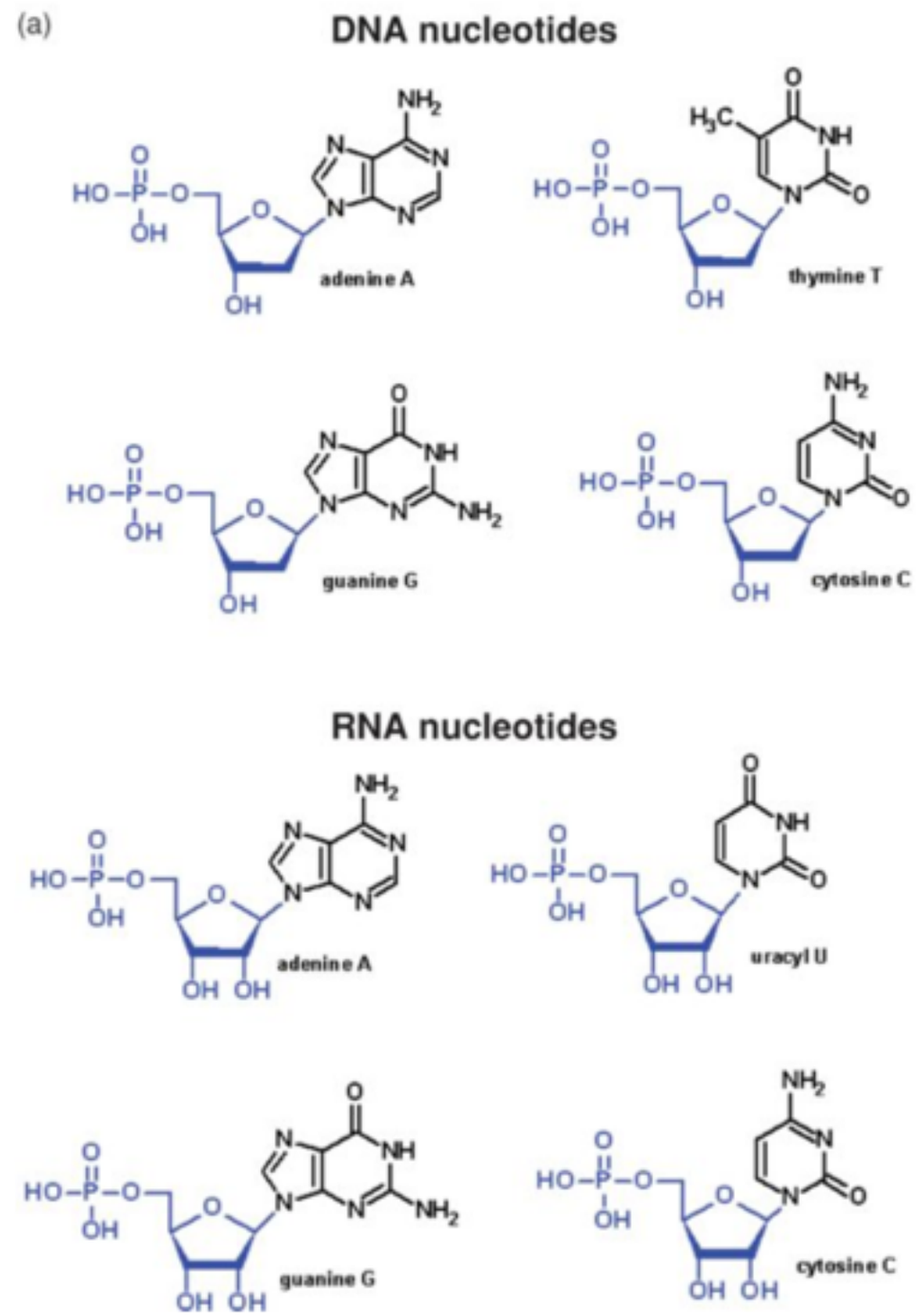
Time-resolved band III spectra of the recovery of CO--myoglobin

Absorption Properties of Nucleotides

Table E1.2. *pH dependence of purine and pyrimidine absorbance maxima*[a]

Base	pH	λ_{max}(nm)
Adenine	1	262.5
Adenine	7	260.5
Adenine	12	269
Cytosine	1	276
Cytosine	7	267
Cytosine	14	282
Guanine	1	248, 276
Guanine	7	246, 276
Guanine	11	274
Thymine	4	264.5
Thymine	7	264.5
Thymine	12	291
Uracyl	4	259.5
Uracil	7	259.5
Uracil	12	284

[a]Data taken from Sober (1997).



- Nucleic acid extinction values are much larger than those of amino acid side-chains close to 280 nm, except for Trp (5600 M^{-1} cm^{-1}) and Tyr (1460 M^{-1} cm^{-1})

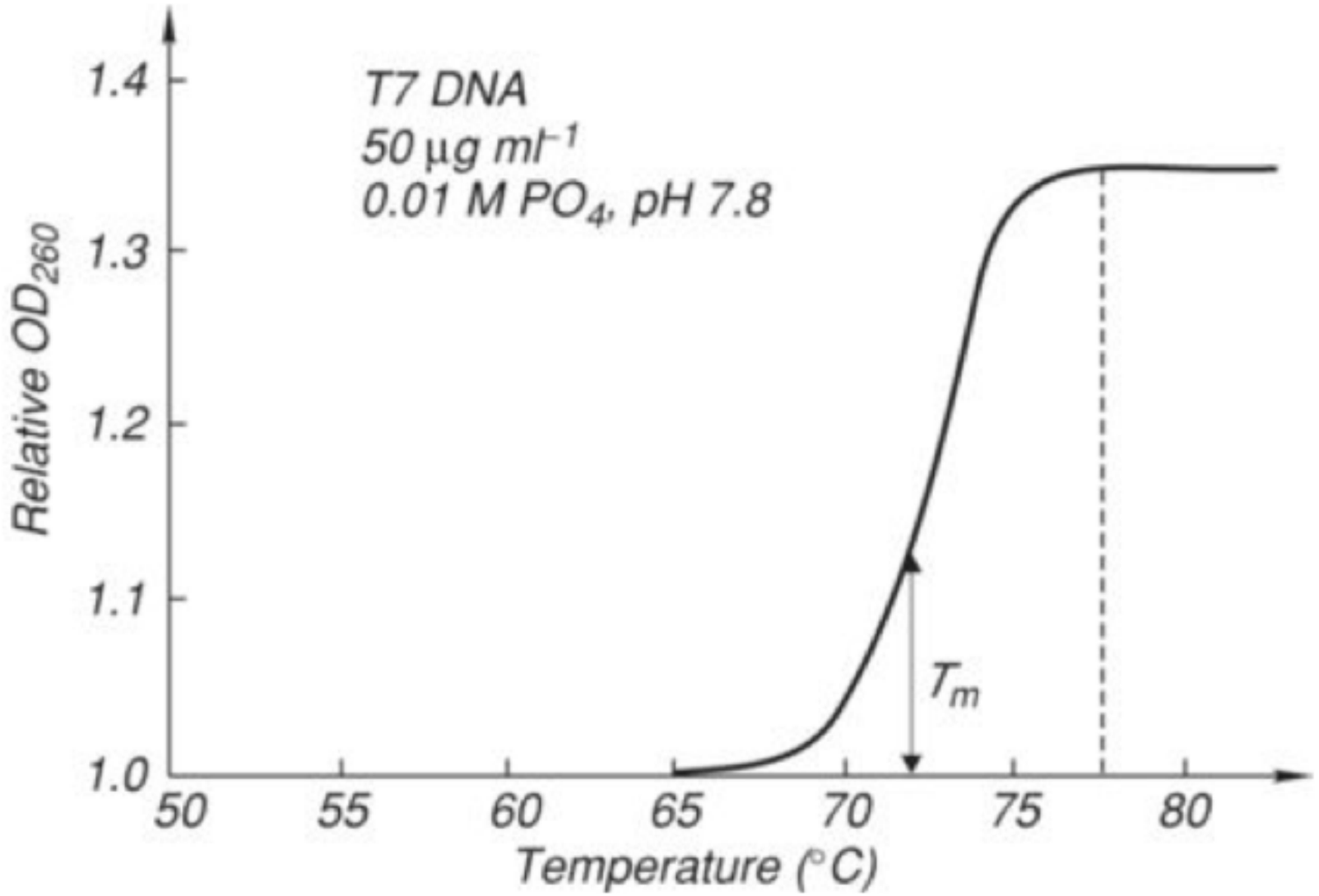
Hyperchromicity

- base, nucleoside, nucleotides, and polynucleotides absorb in the same region
- polynucleotides and nucleic acids absorb less than the same amount of free nucleotides
- stacked bases absorb less than unstacked bases
- due to stacking interactions between adjacent bases in the helical polymer
- ENVIRONMENT MATTERS!!!

Quantification of Nucleic Acids

- Absorbance at 260nm (A_{260})
  - $1A_{260}$ dsDNA=50µg/mL
  - $1A_{260}$ ssDNA=37µg/mL
  - $1A_{260}$ ssRNA=40µg/mL

Effects on UV absorption by hyperchromicity



Infrared Spectroscopy

- Linear IR spectroscopy - study of the structures and interactions of small molecules.
- At first, it appeared to be too ambitious to apply this technique to biological macromolecules, because of their enormous number of vibrational modes.
- Biological macromolecules exhibit an intrinsic order of repeating units:
  - the peptide bond in the protein backbone,
  - the phosphate ester bond
- IR spectra of biological macromolecules are simpler than at first expected
- linear IR spectroscopy provides insights into secondary structure,
- For nucleic acids, information can be obtained on the overall structure and interactions with small molecules such as intercalating drugs or metal ions.
- IR spectroscopy has small energy differences between vibrational and rotational states in molecules related to electric dipole changes

IR Spectroscopy

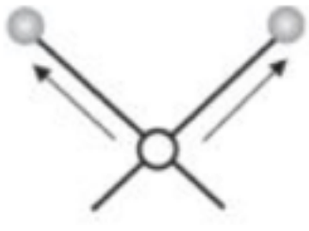
- IR spectroscopy - the frequency of a band is usually expressed in terms of wave numbers in units of cm^{-1}
  - wave number = frequency/velocity
- Divided into near-, mid-, and far-IR regions
- intense absorption of water near 1650 and 3300 cm^{-1} bands, therefore protein samples are measured in D_2O

Table E1.3. *IR spectral regions*

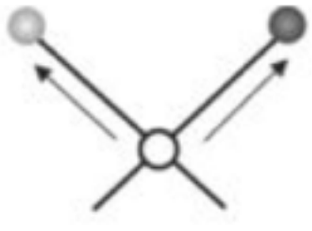
Region	Wavelength range, μm	Wave number range, cm^{-1}	Frequency range, Hz
Near	0.78–2.5	12 800–4000	3.8×10^{14} – 1.2×10^{12}
Middle	2.5–50	4000–200	1.2×10^{14} – 6.0×10^{12}
Far	50–1000	200–10	6.0×10^{12} – 3.0×10^{11}
Most used	2.5–15	4000–670	1.2×10^{14} – 2.0×10^{13}

Types of molecular vibration

Two atom

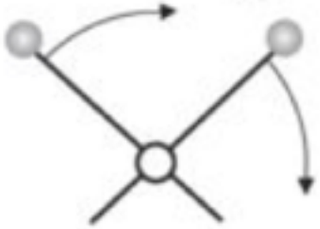


Symmetric

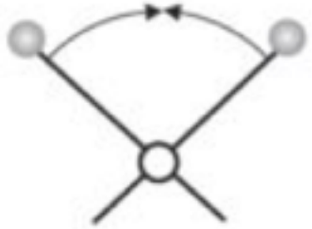


Asymmetric

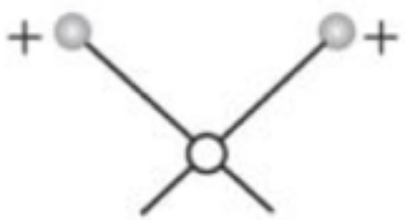
(a) Stretching vibrations



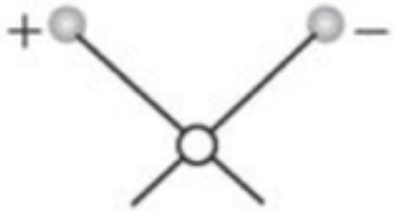
In-plane rocking



In-plane scissoring



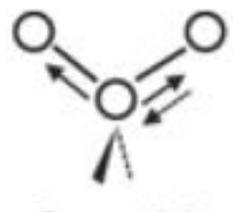
Out-of-plane wagging



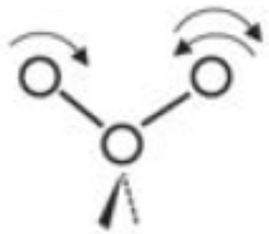
Out-of-plane twisting

(b) Bending vibrations

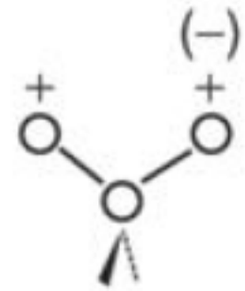
Three atom



Stretching



In-plane bending



Out-of-plane bending

IR-active and IR-inactive modes

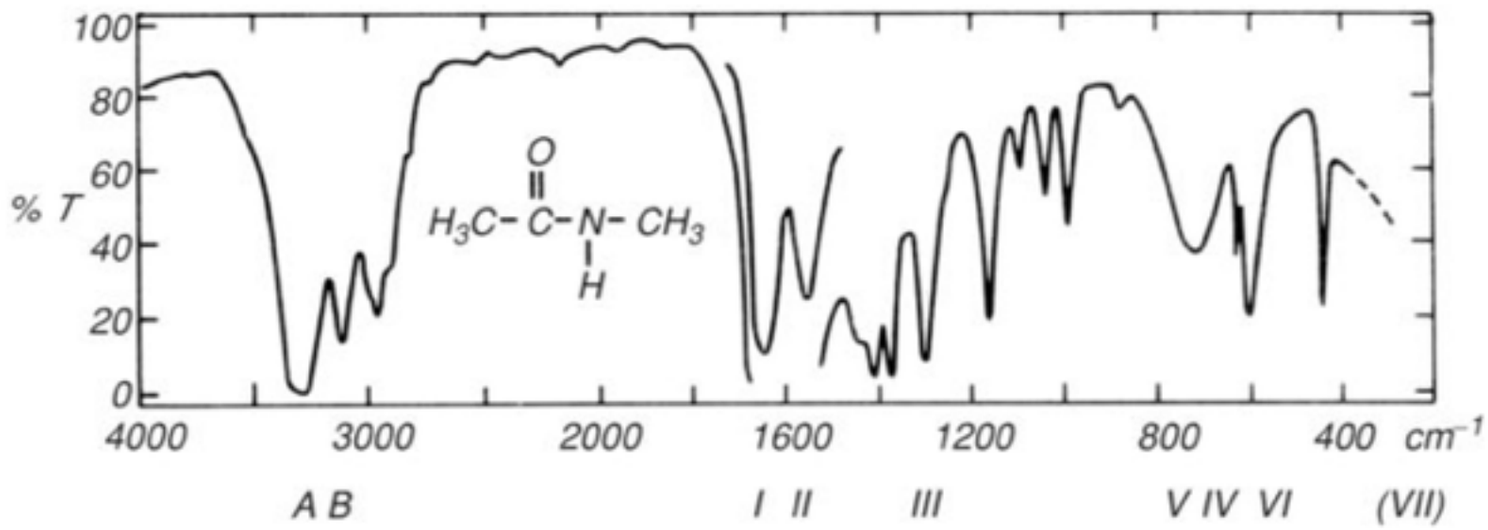
- A permanent dipole moment is associated with a *heteronuclear* diatomic molecule
- *electronegative* values of the bonded atoms are different so that there is a separation between the centers of negative and positive charge
- an oscillating dipole absorbs electromagnetic radiation of the same frequency as its oscillation - IR Active bonds
- mode of vibration that does not give rise to an oscillating dipole moment - IR inactive (*homonuclear* diatomic molecule)
- Is O=C=O IR active? why??

IR of Proteins

- polypeptides and proteins exhibit several relatively strong absorption bands, which are approximately constant in frequency and intensity
- vibrations of the CONH group of the peptide bond. Very prevalent in proteins

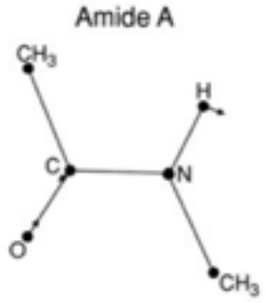
Table E1.4. *Frequency range for characteristic absorption bands of secondary amides in the crystalline state (based on model compounds)*

In-plane modes	
Amide A	~3300 cm^{-1}
Amide B	~3100 cm^{-1}
Amide I	1597–1672 cm^{-1}
Amide II	1480-1575 cm^{-1}
Amide III	1229–1301 cm^{-1}
Amide IV	625–767 cm^{-1}
Out-of-plane modes	
Amide V	640–800 cm^{-1}
Amide VI	537–606 cm^{-1}
Amide VII	~200 cm^{-1}

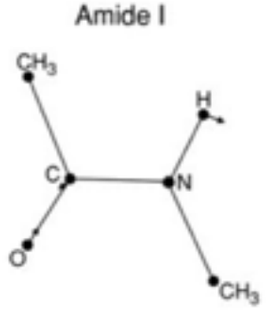


N-methylacetamide is an analogue for the *trans* peptide group

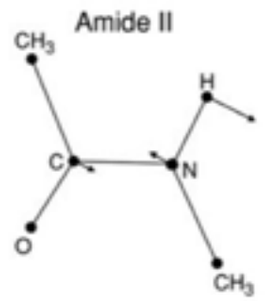
Atomic Displacement of Amides



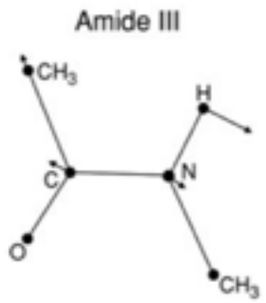
amide A (3400--3460 cm^{-1}) essentially pure N—H no H-boding
amide B (3120--3320 cm^{-1} and 3100 cm^{-1}) similar to Amide A with H-bonding



amide I (C=O stretch, near 1650cm-1)



amide II (N– H bend and C–N stretch, near 1550cm-1)



amide III (C–N stretch, N–H bend, near 1300 cm-1)

IR of Secondary Structure

- Amide I mode (1600--1700 cm^{-1}) of the peptide group is the most widely used band in studies of protein secondary structure.
- Strong IR absorption of water between 1640--1650 cm^{-1}, use D2O solution
  - Concerns??
- Amide II region is relatively strong, it is not very sensitive to secondary structure changes in proteins.
- Bands in the amide III spectral region (1350--1200 cm^{-1}) are predominantly due to the in-phase combination of N--H in-plane bending and C--N stretching vibrations and are highly sensitive to the secondary structure.
- ~(5--10)-fold weaker than those of amide I,

  1330--1295 cm^{-1}, α-helix;
  1295–1270 cm^{-1}, β-turns;
  1270--1250 cm^{-1}, random coils;
  1250--1220 cm^{-1}, β-sheets
- Use amide I and amide III bands to assign secondary structure, compare with known structures

IR of Nucleic Acids

Table E1.6. *Main IR marker bands (in cm^{-1}) of A-, B- and Z-DNA*[a]

Conformation			Assignment
A	B	Z	
1705	1715	1695	In-plane base double stretching
		1433	A-T bases
1418	1425	1408	Deoxyribose
1375	1375		dA•dG anti
		1355	dA•dG syn
1335	1344		dT
1335	1328		dA
		1320	dG
1275	1281		T
		1265	G
1240	1225	1215	Antisymmetric phosphate stretching
1188			Deoxyribose
		1065	Deoxyribose

[a]Data from Taillandier and Liquier (1992).

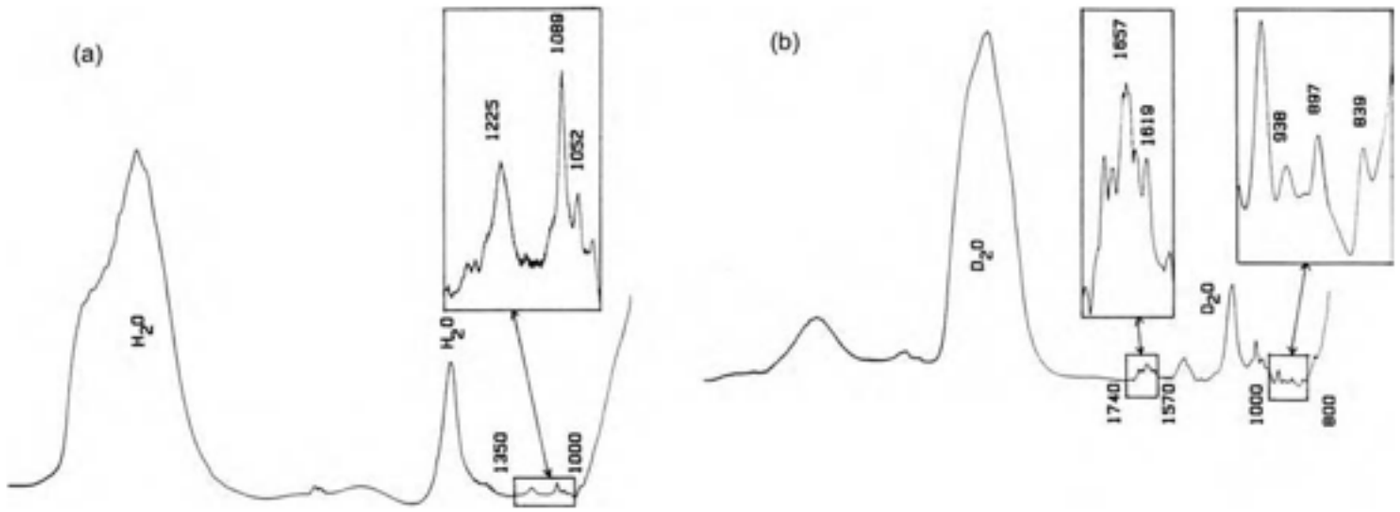


Fig. E1.33 FTIR spectra of DNA in solution: (a) in H_2O solvent; (b) in D_2O solvent. Enlarged parts of the spectra show the absorptions due mainly to the vibrations of the phosphate groups (top), the double bonds of the bases in their plane (bottom left), and the sugar-phosphate backbone (bottom right). (After Taillandier and Liquier, 1992.)

- main vibrations are observed between 1800 and 700 cm^{-1}
- Four types of vibration
- stretching vibrations of double bonds in the base planes (between 1800 and 1500 cm^{-1})
- glycosidic torsion angle in the base sugars (1500 and 1250 cm^{-1})
- strong sugar and phosphate absorption bands (1250 and 1000 cm^{-1})
- vibrations of the phosphodiester main chain (below 1000 cm^{-1})
- Different types of information on DNA structure can be obtained by IR spectroscopy
- Changes in conformation of DNA (e.g. B form to A form)