

## V. Absorption and Emission of Radiation

Consider a beam of radiation passing through a sample. It is characterized by its  $P(\nu)$ , power per unit frequency range.

Absorption of the beam by the sample over a length  $dx$  is written:

$$dP(\nu, x) = -a(\nu)CP(\nu, x) dx \quad (\text{V.1})$$

where  $a(\nu)$  is a frequency-dependent and molecule-dependent constant and  $C$  is the molecular concentration. In the subsequent equations, the  $\nu$  dependence is implicit.

Algebra followed by integration between  $x = 0$  and  $x = l$  yields:

$$\ln \{P(l)/P(0)\} = -aCl \quad (\text{V.2})$$

$$\ln \{P_0/P\} = aCl \quad (\text{V.3})$$

$$A = \log_{10} \{I_0/I\} = \varepsilon Cl \quad (\text{V.4})$$

The intensity  $I$  is radiative power/area and  $A$  is called “absorbance”. Eq. V.4 is the “Beer-Lambert law” and is used in the form  $C = A/\varepsilon l$  to determine concentrations of known molecules.

For proteins,  $A_{280}$  or absorbance at 280 nm is commonly measured to determine protein concentration. The value of  $\varepsilon_{280}$  for a protein is well-approximated with knowledge of the  $\varepsilon_{280}$  values for tryptophan ( $\sim 5600 \text{ cm}^{-1} \text{ M}^{-1}$ ) and tyrosine ( $\sim 1400 \text{ cm}^{-1} \text{ M}^{-1}$ ) and knowledge of the number of tryptophans and tyrosines in the sequence.

For nucleic acids,  $A_{260}$  is the common absorbance and the  $\varepsilon_{260}$ /nucleotide is  $\sim 10000 \text{ cm}^{-1} \text{ M}^{-1}$ .

Experimental determination of  $\varepsilon$  can be made using an analyte solution for which the analyte concentration was determined by some other method. It is also possible in principle to calculate  $\varepsilon$  with knowledge of the energy eigenfunctions of the  $\psi_k \leftarrow \psi_j$  transition. For an electric dipole transition peaked at frequency  $\nu$  with full-width at half-maximum spectral width  $\Delta\nu$ :

$$\varepsilon_{peak} \approx \{(\pi\nu)/[(6.909)\hbar\varepsilon_0c\Delta\nu]\} \times |\langle\psi_k|\boldsymbol{\mu}|\psi_j\rangle|^2 \quad (\text{V.5})$$

If the  $\Delta\nu$  term were brought to left-side of Eq. V.5, this left-side would be  $\varepsilon$  integrated over the entire transition. A particular transition will have a fixed integrated  $\varepsilon$  so that  $\varepsilon_{peak} \propto (\Delta\nu)^{-1}$  and sharper transitions will have higher peak signal relative to broader transitions.

Note  $\varepsilon_{peak} \propto |\langle\psi_k|\boldsymbol{\mu}|\psi_j\rangle|^2 \propto$  probability of photon absorption and  $\varepsilon_{peak} \propto \nu \propto$  absorbed photon energy.

Eq. V.5 does not distinguish between “stimulated absorption” ( $E_k > E_j$ ) and “stimulated emission” ( $E_j > E_k$ ) and  $|\langle \psi_k | \boldsymbol{\mu} | \psi_j \rangle|^2 = |\langle \psi_j | \boldsymbol{\mu} | \psi_k \rangle|^2$ . Rates of stimulated absorption and stimulated emission per molecule are equal:

$$w_{kj} = w_{jk} = (6\varepsilon_0 \hbar^2)^{-1} \times \rho(\nu) \times |\langle \psi_k | \boldsymbol{\mu} | \psi_j \rangle|^2 \quad (\text{V.6})$$

where  $\rho(\nu)$  is the radiant energy per unit frequency per unit volume at the transition frequency (units  $\equiv \text{J/m}^3\text{-Hz}$ ).

The reason for typical net absorption of radiation is that samples are typically near thermal equilibrium and  $N_j > N_k$  where  $N_j$  is the number of molecules in the lower energy state and  $N_k$  is the number of molecules in the higher energy state.

There will be stimulated absorption and emission of radiation in a thermally isolated “black box” system at a constant temperature. The overall rate of stimulated absorption will be greater than the rate of stimulated emission and can be expressed by the inequality  $W_{kj} > W_{jk}$  where  $W_{kj} = N_j w_{kj}$  and  $W_{jk} = N_k w_{jk}$ . This inequality would change  $N_j$  and  $N_k$  and therefore change the temperature which would violate thermal isolation. This paradox can be fixed by inclusion of an additional rate of “spontaneous emission” or emission which occurs in the absence of resonant radiation:

$$A_{jk} = (8\pi^2/3) \times (\nu^3/\varepsilon_0 \hbar c^3) \times |\langle \psi_k | \boldsymbol{\mu} | \psi_j \rangle|^2 \quad (\text{V.7})$$

Spontaneous emission is sometimes reported as the “lifetime”  $\equiv (A_{kj})^{-1}$ . Spontaneous emission is independent of temperature and occurs in the absence of radiation. It therefore always occurs.

Because the spontaneous emission rate is proportional to the cube of the transition frequency, spontaneous emission is most important for electronic transitions at visible and ultraviolet frequencies. This type of spontaneous emission is called fluorescence.

In addition to spontaneous emission, there can be transitions by non-radiative processes, typically collisions with other molecules. The ratio fluorescent photons emitted/photons absorbed is known as the “quantum yield”.

For a typical molecule, electronic absorption and fluorescence are accompanied by excitation of vibrational motion in the electronic excited state (absorption) or electronic ground state (fluorescence). This vibrational excitation is a consequence of the Born-Oppenheimer approximation which states that electronic motion is much more rapid than nuclear motion and that a “vibronic” wavefunction can therefore be considered as a product of a pure electronic and a pure vibrational wavefunction.

Consider absorption from an electronic wavefunction  $\psi_j(\mathbf{r})$  with accompanying vibrational ground state wavefunction  $\chi_{j,v=0}(\mathbf{R})$  with  $\mathbf{r}$  and  $\mathbf{R}$  as the electronic and nuclear coordinates, respectively. The final wavefunction will be a product of a different electronic wavefunction  $\psi_k(\mathbf{r})$  and a different vibrational wavefunction  $\chi_{k,v}(\mathbf{R})$ .

$$\begin{aligned}
\text{Absorption intensity} &\propto |\langle \chi_{k,v}(\mathbf{R}) | \langle \psi_k(\mathbf{r}) | \boldsymbol{\mu} | \psi_j(\mathbf{r}) \rangle | \chi_{j,v=0}(\mathbf{R}) \rangle|^2 \\
&= |\langle \chi_{k,v}(\mathbf{R}) | \langle \psi_k(\mathbf{r}) | e\mathbf{r} | \psi_j(\mathbf{r}) \rangle | \chi_{j,v=0}(\mathbf{R}) \rangle|^2 \\
&= |\langle \psi_k(\mathbf{r}) | e\mathbf{r} | \psi_j(\mathbf{r}) \rangle|^2 \times |\langle \chi_{k,v}(\mathbf{R}) | \chi_{j,v=0}(\mathbf{R}) \rangle|^2
\end{aligned} \tag{V.8}$$

The largest intensities and vibrational integrals will be for similar  $\chi_{j,v=0}(\mathbf{R})$  and  $\chi_{k,v}(\mathbf{R})$ ; i.e. the vibrational wavefunctions with the largest “overlap”.

It is very common that the equilibrium nuclear geometry is different in  $\psi_j(\mathbf{r})$  and  $\psi_k(\mathbf{r})$ . Because  $\chi_{v=0}$  has highest amplitude near  $\mathbf{R}_{eq}$ , there is often little overlap between  $\chi_{j,v=0}$  and  $\chi_{k,v=0}$ . The largest overlap is between  $\chi_{j,v=0}$  and vibrationally excited  $\chi_{k,v}$  with significant amplitude near  $\mathbf{R}_{eq,j}$ . This is known as the “Franck-Condon principle”.

After electronic absorption, there is often rapid non-radiative vibrational relaxation to  $\chi_{k,v=0}(\mathbf{R})$  in the excited electronic state prior to fluorescence. The previously described reasoning predicts that the most intense fluorescence transitions are to vibrationally excited states in the ground electronic state.

Because there is net excitation of vibration in both absorption and fluorescence, the fluorescence spectrum is typically at lower energies relative to the absorption spectrum. The two spectra meet at the  $v = 0 \leftarrow v = 0$  transition.

Detection of fluorescence often has higher sensitivity than detection of absorption because all of the detected fluorescence could in principle be from the molecules whereas detection of absorption is typically done in the presence of background intensity fluctuations of the excitation radiation.

The only amino acid with appreciable fluorescence is tryptophan. The relative magnitude of the tryptophan fluorescence depends on the hydrophilicity of the environment and there is higher tryptophan fluorescence in the hydrophobic interior of the protein and lower tryptophan fluorescence in aqueous solution. For this reason, tryptophan fluorescence is sometimes used to measure the kinetics of protein folding. The tryptophan moves from a more aqueous environment in the unfolded state to a less aqueous environment in the folded state and the fluorescence changes accordingly. The peak fluorescence wavelength also changes from  $\sim 350$  nm to  $\sim 340$  nm.

There is relatively little fluorescence in the visible region from proteins, nucleic acids, and cells. Incorporation of non-native visible fluorophores into specific parts of these systems (e.g. membranes) can be used to follow molecular movement and localization and cellular processes.

“Fluorescence resonance energy transfer (FRET)” is a commonly used method to assess proximity of two molecules or proximity of two regions of a macromolecule. For this method, there are two distinct fluorophores,  $A_1$  and  $A_2$ . The time sequence of the FRET experiment is: (1) excitation of  $A_1$ ; (2) transfer of excitation from  $A_1$  to  $A_2$ ; and detection of  $A_2$  fluorescence.

The absorption spectrum of  $A_1$  is at higher frequency than the absorption spectrum of  $A_2$  so that an excitation frequency can be chosen for which  $A_1$  but not  $A_2$  is excited. For efficient transfer of excitation, the fluorescence spectrum of  $A_1$  should have significant frequency overlap with the absorption spectrum of  $A_2$ . Finally, the fluorescence spectrum of  $A_2$  should be at lower frequency than the fluorescence spectrum of  $A_1$  so that a detection frequency can be chosen for which  $A_2$  but not  $A_1$  fluorescence is detected.

The rate of transfer of excitation between  $A_1$  and  $A_2$  separated by distance  $R$ :

$$\begin{aligned}
k_F &\propto |\langle \psi_1^{ground} | \langle \psi_2^{excited} | \boldsymbol{\mu}_1 \cdot \boldsymbol{\epsilon}_2^{dipole} | \psi_2^{ground} \rangle | \psi_1^{excited} \rangle|^2 \\
&\propto |\langle \psi_1^{ground} | \langle \psi_2^{excited} | (\boldsymbol{\mu}_1 \cdot \boldsymbol{\mu}_2) / R^3 | \psi_2^{ground} \rangle | \psi_1^{excited} \rangle|^2 \\
&\propto R^{-6} |\langle \psi_1^{ground} | \boldsymbol{\mu}_1 | \psi_1^{excited} \rangle \langle \psi_2^{excited} | \boldsymbol{\mu}_2 | \psi_2^{ground} \rangle|^2
\end{aligned} \tag{V.9}$$

There is typically competition between the  $A_1 \rightarrow A_2$  excitation transfer and  $A_1$  fluorescence and non-radiative relaxation. The overall rate of these other processes is denoted “ $k_Q$ ”. The  $A_2$  fluorescence intensity  $F_2$  (typically measured as photons/time or power  $\equiv$  energy/time) will depend on the relative magnitudes of  $k_F$  and  $k_Q$ :

$$F_2 = b \times [k_F / (k_F + k_Q)] \tag{V.10}$$

The “ $b$ ” in Eq. V.10 is a constant which reflects the rate of  $A_1$  excitation as well as the “quantum yield” probability that  $A_2$  will fluoresce after excitation rather than relax non-radiatively. This constant can often be experimentally determined. The  $k_F$  and  $k_Q$  are defined:

$$k_F = c R^{-6} \tag{V.11}$$

$$k_Q = c R_0^{-6} \tag{V.12}$$

Eq. V.10 is written:

$$\begin{aligned}
F_2 &= b \times [R^{-6} / (R^{-6} + R_0^{-6})] \\
&= b \times [1 + (R/R_0)^6]^{-1}
\end{aligned} \tag{V.13}$$

$R_0$  is the  $A_1$ - $A_2$  distance at which  $F_2$  has half-maximal magnitude and can often be determined experimentally. A typical value of  $R_0$  is 50 Å. The ratio  $F_2/b$  is known as the “FRET efficiency” and varies between 1 ( $R \approx 0$ ) and 0 ( $R \gg R_0$ ). In addition to the potential for relatively precise determination of  $R$  afforded by FRET measurements, Eq. V.13 shows that there is a very strong dependence of  $F_2$  on  $R$  with  $F_2(R = 2R_0) / F_2(R = R_0) \approx 0.03$ . Because typical  $R_0$  are of the same magnitude as the sizes of proteins and small nucleic acids, FRET is useful for qualitative detection of specific protein/protein and protein/nucleic acid binding.  $A_1$  would be attached to one molecule and  $A_2$  would be attached to the potential binding partner molecule. Detection of appreciable  $F_2$  would be evidence for binding.