Chem 728 Lecture Notes

The following are lecture notes for Chem 728 (by C. Martin Fall 1995, with minor modifications by L. Thompson). Much of the material is taken directly from the indicated references (old vH, some refs updated to new). This is not intended to replace the original references, but is made available solely for the convenience of students in the class.

Review of Thermodynamics

See van Holde, Chapter 2

As chemists we're in terested in molecules - the microscopic view... Thermodynamics takes a macroscopic view of the behavior of large numbers of molecules (and thus it can take advantage of statistical behavior) to deduce a great deal about a reaction without needing to know its microscopic details. This understanding can then provide important insight into the molecular level. We'll see how... **>>example.**

Definitions

Thermodynamics - describes distribution of energy within (states of) a system.

System - any subset of the universe having spatial boundaries

Transfer of matter between system and universe:

open - transfer of matter allowed

closed - transfer of matter not allowed

Transfer of heat between system and universe

non-adiabatic - heat allowed (eg. eppendorf tube floating in a large water bath)

adiabatic - insulated, no transfer of heat (eg. (approx) eppendorf tube placed in styrofoam insulation)

State of a system - defined only at equilibrium

Specified by two of (T,P,V) plus the masses and identities of all chemical species

Properties of states of the system

Extensive - requires for their definition the full specification of the system including masses and identities of components

examples - volume, energy, entropy - double the number of molecules, double the volume

Intensive - only relative amounts of components need be known

examples - density, viscosity - double the number of molecules, no change in density

Changes between states

Reversible - path from initial to final state proceeds through a succession of “near-equilibrium” states

Irreversible - NOT above

State variables - path independent

This is quite important for calculations, particularly for complex processes: a simpler path connecting the same 2 states can be used to calculate path-independent quantities (typically all but q and w).

Energetics

q - Heat → energy supplied **to** the system as **heat**

energy transferred into or out of a system as a consequence of a temperature difference between the system and surroundings

heat absorbed *by* the system is *positive*

w - Work → energy supplied **to** the system as **work**

any exchange of “**non-heat**” energy between the system and its surroundings. Examples:

volume change under constant pressure ($P\Delta V$)

changes in surface area under constant surface tension (2D analog of $P\Delta V$)

electrical work, etc.

work done *on* the system is *positive* - Beware: this convention is **NOT** universal! ie van Holde opposite

E or **U** - Internal Energy (extensive state function)

The energy *within* the system. For our purposes, only energy which can be modified by chemical processes (eg. we ignore nuclear energy).

examples - translational, vibrational, and rotational energy of the molecules, chemical bond energy, nonbonding interactions (eg. dipolar, ionic)

Typically defined *relative to* a standard state. Only *change* in E is usually considered.

H - Enthalpy (state function)

H = E + PV Enthalpy is the sum of the internal energy plus the product of the system volume times the external pressure

The First Law - "Energy is conserved"

$\Delta E = q + w$ $dE = dq + dw$

"The change in the internal energy of a system is the total heat *absorbed by* the system plus the total work *done on* the system by its surroundings"

Note that E is a state variable, but that q and w are not (some texts note this with different symbol for dq, dw).

If only PΔV work is possible then $dw = -PdV$

$dE = dq + dw = dq - PdV$

Note: one must apply work **to** the system (**dw > 0**) in order to compress the system (**dV<0**)

For convenience, define enthalpy, $\Delta H = \Delta(E + PV)$

$dH = d(E + PV) = dE + PdV + VdP = dq + dw + PdV + VdP$

If only PΔV work is possible then $dw = -PdV$

$dH = dq + VdP$

Limiting conditions:

At constant volume: $dE = dq$ $(\Delta E = q_v)$

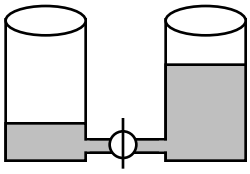
At constant pressure: $dH = dq$ $(\Delta H = q_p)$

Since most biological processes occur under constant pressure (1 atm), ΔH is the term of choice to measure the energy of the system. However, since volume changes in most biological processes are small, $\Delta H \approx \Delta E$, so the distinction is minor.

>> Review these ideas by following the reasoning of van Holde Fig 2.1

The Molecular View - Statistical thermodynamics

From our point of view, a system is really just a collection of molecules. It is the individual actions and energies of the molecules that determine the properties of the system. Why then, do we often ignore the individual players? The answer is that the statistical behavior of very large numbers of well-behaved particles near equilibrium can be very accurately predicted, even though the exact behavior of any single particle cannot be predicted.



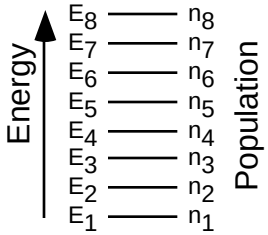
Intuitive example: consider two beakers containing a liquid and connected by a tube with a closed stopcock in between. You can readily predict what will happen when you open the stopcock. Fluid will flow (individual molecules will move from one container to the other) until the liquid levels are equal. It doesn't matter whether the fluid is water, honey, or benzene, the final state will be the same. Similarly, it doesn't matter what diameter the connecting tube is, or how long the connector is. Although the kinetics of approach to equilibrium may depend on these parameters, the final state will not. In contrast, you cannot predict *which* individual molecules will end up in which container (in fact the disposition of an individual molecule will vary with time, even at equilibrium).

Thermodynamics is really concerned with how energy is *distributed* over a very large number of particles. In a gas or a liquid, different particles can have different energies. These energies are statistically distributed among the particles and the sum of their individual energies is the total energy of the system.

Equilibrium is the most probable distribution of energies.

Using statistical mechanics it is possible to derive an equation known as the Boltzmann equation, which relates the relative (statistical) populations of non-degenerate states ("states" available to the particles) to their energy levels. For two such states, *i* and *j*, we have:

$$\frac{n_i}{n_j} = e^{-\frac{(\varepsilon_i - \varepsilon_j)}{k_B T}}$$

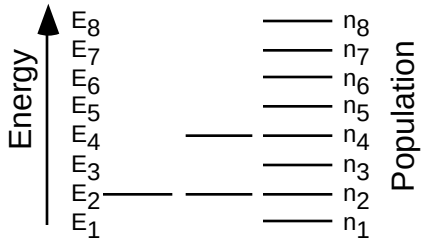


This equation defines the *most probable* (ie. equilibrium) distribution for the system.

The above assumed non-degenerate particle states. In other words, no two particle states may have the same energy. Since we are dealing with simple statistical distributions, if a given particle energy exists for two different particle states, then that energy will be more often represented in the statistical distribution than an energy represented by one state.

To include the general concept of degeneracy, the more general form of the Boltzmann equation is written:

$$\frac{n_i}{n_j} = \frac{g_i}{g_j} e^{-\frac{(\varepsilon_i - \varepsilon_j)}{k_B T}}$$



where each energy level, E_i, contains g_i degenerate states, and n_i is the number of particles with energy E_i. This explains the ratio of the populations of two energy levels. To determine the population of one state relative to all possible states, we have

$$\frac{n_i}{N} = \frac{g_i e^{-\frac{(\varepsilon_i - \varepsilon_j)}{k_B T}}}{\sum_l g_l e^{-\frac{(\varepsilon_l - \varepsilon_j)}{k_B T}}}$$ (N.B.: *j* is simply an arbitrary reference state for the calculation)

The above equation is known in statistical mechanics as the molecular partition function. Remember well the basis that thermodynamics has in statistics.

Second Law of Thermodynamics

How do we know what *direction* a reaction will take? A reaction will go towards the *most probable* state (overall distribution of energies) for the system. From statistics again, we can look at the two distributions below for three degenerate energy levels and predict which is more likely.

A: B:

There is only one way to distribute the particles to get state A. There are a lot more ways of distributing particles to get state B (11!/(4! 4! 3!)=11,550). If A and B have equal energies, B will occur much more often. Another way of saying this is that *there is more randomness in distribution B* than in distribution A. Given this, we need to have a way to express "randomness."

From probability theory, we know that the number of ways of arranging N distinguishable particles with n_1 in one group, n_2 in another group, etc. is

$$W = \frac{N!}{n_1! n_2! n_3! \ldots n_n!}$$ where $N = n_1 + n_2 + n_3 + \ldots + n_n$

Define entropy: $S = k \ln W$ **(an *extensive* property)**

where k is the Boltzmann constant, k_B (from here forward, we will drop the subscript "B" except when it is necessary for clarity), and W is the number of ways in which the individual particle states can be distributed within a particular energy level.

Note that S is *extensive*, such that for a system composed of two parts, 1 and 2, the total entropy is given by:

$$S = S_1 + S_2 = k \ln W_1 + k \ln W_2 = k \ln(W_1 W_2)$$

in other words, $W = W_1 W_2$, which is expected from statistics: if the number of ways of distributing particles in state 1 is W_1 and the number of ways for part 2 is W_2, then the number of ways of distributing particles in the combined state is simply $W_1 W_2$.

Similarly, we can ask about the change in entropy of a system initially characterized by a distribution W_o going to a distribution W_f

$$\Delta S = S_f - S_o = k \ln W_f - k \ln W_o = k \ln \left(\frac{W_f}{W_o} \right)$$

expanding our definition of entropy,

from $W = \frac{N!}{n_1! n_2! n_3! \ldots n_n!}$ we expand $\ln W = \ln(N!) - \ln(n_1!) - \ln(n_2!) - \ln(n_3!) \ldots - \ln(n_n!)$

but note that for large n (Stirling's approximation): $\ln(n!) \approx n \ln(n) - n$

$$\ln W = (N \ln(N) - N) - (n_1 \ln n_1 - n_1) - (n_2 \ln n_2 - n_2) \ldots - (n_n \ln n_n - n_n)$$

$$\ln W = N \ln N - n_1 \ln n_1 - n_2 \ln n_2 \ldots - n_n \ln n_n - N + n_1 + n_2 \ldots + n_n$$

$$\ln W = N \ln N - n_1 \ln n_1 - n_2 \ln n_2 \ldots - n_n \ln n_n$$

(remember that $N = n_1 + n_2 + n_3 + \ldots + n_n$). Finally we can summarize:

$$\ln W = N \ln N - \sum_i n_i \ln n_i$$

Now let's look at a change of the system in which we redistribute some particles to different energy levels, and we'll do it in infinitesimally small steps. From before, S = k lnW, so that:

$$dS = kd(\ln W) = kd\left(N \ln N - \sum_i n_i \ln n_i \right)$$

$$= kd(N \ln N) - k \sum_i d(n_i \ln n_i)$$

$$= 0 - k \sum_i \left[d(n_i) \ln n_i + n_i d(\ln n_i) \right] = -k \sum_i \left[dn_i \ln n_i + n_i \frac{dn_i}{n_i} \right] = -k \sum_i dn_i \ln n_i - k \sum_i dn_i$$

The summation in the last term represents the sum of all population changes in all levels. Since the N particles are restricted to the levels 1 through n (*ie.*, particles can only redistribute among the levels), the sum of all changes is simply 0.

If this causes you trouble, imagine that if you move 5 particles from level 3 to level 6. For this transfer dn_3=-5 and dn_6=+5. As for this example, the sum of all transfers is simply 0.

Therefore $dS = -k \sum_i \ln n_i dn_i$

remembering the Boltzmann distribution, $\frac{n_i}{n_j} = e^{-\frac{(\varepsilon_i - \varepsilon_j)}{k_B T}}$

$$\ln n_i = \ln n_j - \frac{(\varepsilon_i - \varepsilon_j)}{k_B T} = \ln n_j - \frac{\Delta \varepsilon_j}{kT}$$

Placing this into the equation for dS above

$$dS = -k \sum_i \left(\ln n_j - \frac{\Delta \varepsilon_i}{kT} \right) dn_i = -k \ln n_j \sum_i dn_i + k \sum_i \frac{\Delta \varepsilon_i}{kT} dn_i$$

$$dS = 0 + k \sum_i \frac{\Delta \varepsilon_i}{kT} dn_i = \frac{1}{T} \sum_i \Delta \varepsilon_i dn_i$$

Let's think again about the summation of $\Delta\varepsilon_i$: we are talking about redistributing particles between various levels. The summation totals the changes in energies, weighted by the number of particles making each change. If *more particles move to higher energies* than move to lower energies, the system must *absorb heat* (and vice versa). Hence, the summation is simply the amount of heat absorbed by the system, q_{rev}.

$$dS = \frac{1}{T} \sum_i \Delta \varepsilon_i dn_i = \frac{dq_{rev}}{T} \quad \text{or} \quad \Delta S = \int \frac{dq_{rev}}{T} = \frac{q_{rev}}{T}$$

In general then, we can directly calculate S for a process *if* we can define q_{rev} along a *reversible* path from state A to state B. You will remember that since S is a state function, we can exploit this result even if the actual path taken in an experiment is not reversible. A simple example using the expansion of an ideal gas is given in van Holde (pp. 80-81).

This is then expanded to include mixing two solutions. Our intuition says that the entropy of the final mixture will be greater than the sum of the entropies of the two pure components (each particle has more choices available to it).

Again intuition tells us that the melting of a crystalline solid or the unfolding of a protein (but see important discussion below) will lead to an increase in entropy.

$$\Delta S = \frac{q_{rev}}{T} = \frac{\Delta H_{melting}}{T_{melting}}$$

In general, heating of a sample will lead to an increase in entropy, and this can be seen from the Boltzmann analysis above. Heating a sample must lead to increased population of higher particle energy levels. This results in a larger distribution of particle energies, and therefore to increased entropy.

Define heat capacity, C_p

The change in the internal heat of a system as a function of temperature (and at constant pressure) can be expressed as:

$$\left(\frac{\partial q_{rev}}{\partial T}\right)_p = C_p$$

Remembering that $dS = \frac{dq_{rev}}{T}$

$$dS = \frac{C_p dT}{T} \qquad \Delta S = \int_{T_1}^{T_2} \frac{C_p dT}{T}$$

Assuming (and this is an assumption) that the heat capacity is not temperature dependent over the temperature range from T_1 to T_2, then

$$\Delta S = C_p \int_{T_1}^{T_2} \frac{dT}{T} = C_p \ln\left(\frac{T_2}{T_1}\right)$$

Re-examine "equilibrium"

From above we have $\partial q_{rev} = T\partial S$, and returning to our definition of E we have

$$dE = dq_{rev} + dw$$
$$= TdS - PdV$$ at constant pressure.

For a reversible process at equilibrium, the internal energy E and the system volume V are constant, so that dS must be zero. If we reversibly perturb the system in an infinitesimally small manner, dS around equilibrium is zero. This means that S must be in a local maximum or local minimum.

From our previous statements about most probable states, we conclude that an isolated system (E and V constant), will be at equilibrium only when the entropy is maximum (minimum entropy would correspond to few ways of arranging the particles).

Finally state the Second Law: An isolated system will approach a state of maximum randomness - directionality.

A more relevant term for biological systems - ΔG (constant T & P)

We rarely encounter isolated systems at constant volume. Instead most of life's processes occur under constant temperature (*eg.* physiological 37°C or room temperature ≈25°C) and constant pressure (1 atm). We introduce the Gibbs free energy, as a function of T and P

$G = H - TS$

$H = E + PV$

$dG = dH - d(TS) = dE + VdP + PdV - SdT - TdS$

for a reversible process $dE = TdS - PdV$, so that

$dG_{rev} = VdP - SdT$

If we consider ***equilibrium at constant temperature and pressure***, then $\underline{dG = 0}$

Just as entropy reaches a maximum at equilibrium, G reaches a minimum.

Also note the more familiar expression at constant temperature $\Delta G = \Delta H - T\Delta S$

Back to Boltzmann

——— *i*

——— *j*

Consider two energy levels, *i* and *j*,

From before we had $\frac{n_i}{n_j} = \frac{g_i}{g_j} e^{-\frac{(\varepsilon_i - \varepsilon_j)}{k_B T}}$

But g_i is the degeneracy, or number of ways of attaining energy ε_i, which is W_i

$$\frac{n_i}{n_j} = \frac{W_i}{W_j} e^{-\frac{(\varepsilon_i - \varepsilon_j)}{kT}} = e^{\ln\left(\frac{W_i}{W_j}\right)} e^{-\frac{\Delta\varepsilon}{kT}} = e^{\ln W_i - \ln W_j - \frac{\Delta\varepsilon}{kT}} = e^{\left(\frac{kT \ln W_i - kT \ln W_j - \Delta\varepsilon}{kT}\right)}$$

If we move from particle energy ($\Delta\varepsilon$) to a molar energy (ΔE), also replacing k by R, and then approximate $\Delta E \approx \Delta H$, we have

$$\frac{n_i}{n_j} = e^{\left(\frac{TS_i - TS_j - \Delta H}{RT}\right)} = e^{-\left(\frac{\Delta H - T\Delta S}{RT}\right)} = e^{-\frac{\Delta G}{RT}}$$

You will of course notice that the left side is just the equilibrium constant for a reaction going from state j to state i. This then leads us to a familiar expression

$$\frac{n_i}{n_j} = K = e^{-\frac{\Delta G}{RT}} \quad \text{or} \quad \ln K = -\frac{\Delta G}{RT}$$

Aside: The text (van Holde) first talks about the unfolding of a protein as necessarily having an associated increase in entropy, as a result of the increased motional freedom of the polypeptide and the side chains. This is what most people would come to, looking at the protein as an isolated system. At the end of the chapter, van Holde discusses the real situation - protein unfolding may actually have an *unfavorable* entropic component. **Why?** The answer is that it is unrealistic to treat an isolated protein as a system unto itself. In fact, in solution a protein is interacting substantially with the solvent (water) and components of the solvent. Indeed, it is these interactions which lead to stable folding of proteins. When we draw the line for our system, we must include these interactions.

A charged or polar group on the surface of a protein will "want to" interact with a charge or polar group in solution. Even if a direct interaction with a salt ion is formed, water will ultimately be involved in forming interactions with the polar group(s). Hence, the exposure of a charged or polar group on the surface of the protein serves to orient (restrict the motion of) water molecules - reducing their entropy. Of course, water molecules will also interact with themselves, but the interactions are transient and more varied (large entropy). Even more so, exposure of a nonpolar group to solvent forces a preferred orientation on associated solvent molecules (water) reducing the entropy of the system.

When a protein folds up, many of these interactions are satisfied internally (a polar side chain might interact with a backbone carbonyl). As before the entropy associated with each group decreases, but it was already low since the two portions of the molecule are ultimately covalently linked. Since this decrease in entropy is less than the entropy loss associated with solvent caging, the total entropy (disorder) of the system can actually be

higher in the system containing a folded protein. We will discuss this concept at greater length in future lectures.

Brief Aside:

Introduction to MathCAD

When you first run MathCAD you will be placed onto an empty document.
You can simply click anywhere with the mouse to type an equation.
To define a variable, use :=
eg. a:=3.0 YVal:=a/5 (this will then convert to a more familiar form)
Note that expressions on a page are evaluated left to right and top to bottom, so that the second expression above must be located to the right of or below the left expression, otherwise "a" will be undefined.
To check the value of a variable, use =
eg. after the above, type YVal= the screen will respond with YVal = 0.6
Any time that the "insertion point" is in a variable, typing will do as you expect.
When you want to "operate on" a variable, you often have to "back up" and select the whole variable first. To do this type either the "space bar" or the "up arrow key" while the insert point is anywhere in the name of that variable (you may have to click to get it there). You will see the single selection mark change to a rectangle surrounding the variable.
If you type "space bar" or "up arrow key" more than once, more variables will be enclosed in your rectangle for selection.
To delete a selection, you must type "Command-X" (or choose "Cut" from the edit menu).
For some strange reason, the "delete key" will not delete.

Error Analysis

For an observable f(x,y,z) which is a function of variables x, y, and z, each with inherent error Δx, Δy, and Δz, respectively

$$\Delta f(x,y,z) = \sqrt{\left(\frac{\partial f(x,y,z)}{\partial x}\right)^2 \Delta x^2 + \left(\frac{\partial f(x,y,z)}{\partial y}\right)^2 \Delta y^2 + \left(\frac{\partial f(x,y,z)}{\partial z}\right)^2 \Delta z^2}$$

L'Hopital's Rule

What to do in the limit as a function goes to either 0/0 or ±∞/±∞.

Put simply: $\lim_{x \to c} f(x)/g(x) \rightarrow \left[\infty/\infty \text{ or } 0/0\right] \rightarrow \lim_{x \to c} \frac{\partial f(x)}{\partial x} \Big/ \frac{\partial g(x)}{\partial x}$

Dimensional Analysis

When doing derivations, it is very easy to drop a term or part of a term. An easy way to find such errors is to remember that most, if not all, parameters in a function have ***units***. Consequently, it doesn't make sense to add an expression in units of concentration to an expression in units of concentration per time. So at the end of a derivation, make sure that in any sum or difference expression, all additive terms have the same units. If they don't, then you can be certain that you made an error. To find the error, repeat the dimensional analysis on each preceding expression in your derivation.
An obvious corrollary of this is to always make sure that the final result has the correct units. If you're solving for velocity, and the expression you end up with doesn't have time in the denominator, then you are in trouble.

Solution thermodynamics

Definition: Solution - a single phase system containing more than one *independently variable* substance (component). At equilibrium, the solution can be described completely by specification of the components and knowledge of their interactions. Extensive and intensive variables depend on the composition.

Definition: Partial molar/specific quantities - for any extensive property X, the corresponding partial molar quantity is

$$\overline{X}_i = \left(\frac{\partial X}{\partial n_i}\right)_{T,P,n_{j\neq i}}$$

In other words, it is the differential change in the extensive property X associated with the differential change in the molar amount of species *i*, holding all else constant. It may very well be a function of the other parameters (T,P, the amounts and identities of other components).

Partial *specific* quantities are the same, but are with respect to the differential change in *mass* of the component.

As an example, if you add volume x of a solute to volume y of a solution, the resulting volume is not necessarily x+y. The volume change is governed by the partial specific volume of the solute in that solution.

Note that the observable, the total extensive quantity, is sum of the products of all partial specific quantities multiplied by the number of moles of each:

$$X = \sum_{i=1}^{n} n_i \overline{X}_i \quad \text{or} \quad dX = \sum_{i=1}^{n} \overline{X}_i dn_i$$

One extensive property that deserves expansion is the free energy, $\overline{G}_i$, the partial molar free energy (also known as the chemical potential, μ_i.

$$\overline{G}_i = \mu_i = \left(\frac{\partial G}{\partial n_i}\right)_{T,P,n_{j\neq i}} \qquad G = \sum_{i=1}^{n} n_i \mu_i$$

Just as we can look at the dependence of an extensive variable on the molar amount of a species, we can also look at its dependence on other solution parameters. In general:

$$dG = \left[\left(\frac{\partial G}{\partial T}\right)_{P,n_i}\right] dT + \left[\left(\frac{\partial G}{\partial P}\right)_{T,n_i}\right] dP + \sum_{i=1}^{n} \left[\left(\frac{\partial G}{\partial n_i}\right)_{T,P,n_{j\neq i}}\right] dn_i$$

from before we had: $dG = -SdT + VdP$

which is now more generally:

$$dG = -SdT + VdP + \sum_{i=1}^{n} \left[\left(\frac{\partial G}{\partial n_i}\right)_{T,P,n_{j\neq i}}\right] dn_i = -SdT + VdP + \sum_{i=1}^{n} \mu_i dn_i$$

Since we often restrict ourselves to constant T and P, then $dG = \sum_{i=1}^{n} \mu_i dn_i$

An important result of this can be seen, backing up to the more general $G = \sum_{i=1}^{n} n_i \mu_i$

we have $dG = \sum_{i=1}^{n} n_i d\mu_i + \sum_{i=1}^{n} \mu_i dn_i$

but since at constant temperature and pressure $dG = \sum_{i=1}^{n} \mu_i dn_i$ then $\sum_{i=1}^{n} n_i d\mu_i = 0$

The simple result of this is that for an n-component system, μ_i for only n-1 components are independent (Gibbs-Duhem).

Also, the differential free energy of a solute in a system of two phases (1 and 2) at equilibrium and at constant T and P is

$$dG = \sum_{i=1}^{2} \mu_i dn_i = \mu_1 dn_1 + \mu_2 dn_2 = 0$$

If we transfer a small amount of the solute from phase 1 to phase 2, $dn_1 = -dn_2$, so that

$-\mu_1 dn_2 + \mu_2 dn_2 = 0 \qquad \mu_1 = \mu_2$

In other words, for a solute at equilibrium between two phases, the chemical potential for that species must be the same in both phases.

Since μ_i is related to concentration, c_i, this will lead to rules for equilibria.

Ideal Solutions

An ideal solution is one in which all components obey Raoult's law, relating vapor pressure to mole fraction, X_i.

Alternatively viewed, an ideal solution is one for which the enthalpy of mixing is 0 and the only source of energy change is due to changes in the *entropy* of mixing. It implies a lack of interactions between solutes, in exactly the same way that the ideal gas law ignores molecule-molecule interactions.

From above, we had $S = k \ln W = k \ln \dfrac{N_0!}{N_1! N_2! \ldots N_n!}$

Using Stirling's approximation as before,

$$\Delta S_{mixing} = -k \sum_{i=1}^{n} N_i \ln \frac{N_i}{N_0} = -k \sum_{i=1}^{n} N_i \ln X_i \quad \text{where} \quad X_i = \frac{N_i}{N_0}$$

Therefore $\Delta S_{mixing} = -R \sum_{i=1}^{n} n_i \ln X_i$

$$\Delta G_{mixing} = \Delta H_{mixing} - T\Delta S_{mixing} = 0 - T\Delta S_{mixing} = RT \sum_{i=1}^{n} n_i \ln X_i$$

but $G = \sum_{i=1}^{n} n_i \mu_i$

If we define $\Delta G_{mixing} = G_{soln} - \sum_{i=1}^{n} G_i^{pure} = \sum_{i=1}^{n} n_i \mu_i - \sum_{i=1}^{n} n_i \mu_i^0 = \sum_{i=1}^{n} n_i \left(\mu_i - \mu_i^0\right)$

Combining ΔG_{mixing} from the two approaches

$$\Delta G_{mixing} = \sum_{i=1}^{n} n_i \left(\mu_i - \mu_i^0\right) = RT \sum_{i=1}^{n} n_i \ln X_i$$

therefore

$\mu_i - \mu_i^0 = RT \ln X_i$

van Holde then discusses that for dilute solutions, $X_i \approx C_i$, so that for most purposes:

$\mu_i = \mu_i^0 + RT \ln C_i$

For a reaction: aA + bB <--> cC + dD

$$G = \sum_{i=1}^{n} n_i \mu_i$$

$\Delta G = G_{final} - G_{initial}$ but

$\Delta G = c\mu_C + d\mu_D - a\mu A - b\mu_B$

$\Delta G = \left(c\mu_C^o + cRT \ln C_C\right) + \left(d\mu_D^o + dRT \ln C_D\right) - \left(a\mu_A^o + aRT \ln C_A\right) - \left(b\mu_B^0 + bRT \ln C_B\right)$

$\Delta G = \left(c\mu_C^o + d\mu_D^o - a\mu_A^o - b\mu_B^0\right) + \left(cRT \ln C_C + dRT \ln C_D - aRT \ln C_A - bRT \ln C_B\right)$

$$\Delta G = \Delta G^o + RT \ln \frac{C_C^c C_D^d}{C_A^a C_B^b}$$ a classic!

At equilibrium $\Delta G = 0$, so

$$\Delta G^o = -RT \ln \frac{C_C^c C_D^d}{C_A^a C_B^b} = -RT \ln K_{eq}$$ another classic!

Also $\Delta G^o = \Delta H^o - T\Delta S^o = -RT \ln K_{eq}$ so $\ln K_{eq} = \frac{-\Delta H^o}{RT} + \frac{\Delta S^o}{R}$

If ΔH^o and ΔS^o are independent of temperature (often NOT for protein interactions, but often is OK over a narrow temperature range), then the temperature dependence of K_{eq} can yield ΔH^o and ΔS^o.

van't Hoff equations

In calorimetry, one can directly measure heats associated with certain types of reactions.

Let's continue to look at the temperature dependence of some thermodynamic parameters.

Starting with the expression: $\ln K_{eq} = \frac{-\Delta H^o}{RT} + \frac{\Delta S^o}{R}$

We can derive the differential expression: $\left(\frac{\partial \ln K_{eq}}{\partial (1/T)}\right)_P = \frac{-\Delta H^o}{R}$

or alternatively, $\left(\frac{\partial \ln K_{eq}}{\partial T}\right)_P = \frac{\Delta H^o}{RT^2}$

These are various forms of the van't Hoff relation.

We can also predict the temperature dependence of the equilibrium constant over a finite change in temperature:

$\ln K_{T_i} = \frac{-\Delta G^o_{T_i}}{RT_i}$ leads to $\ln K_{T_2} - \ln K_{T_1} = \frac{-\Delta G^o_{T_2}}{RT_2} - \frac{-\Delta G^o_{T_1}}{RT_1}$

or $\ln K_{T_2} = \ln K_{T_1} + \frac{-\Delta G^o_{T_2}}{RT_2} - \frac{-\Delta G^o_{T_1}}{RT_1}$

We can also express this in terms of ΔH and ΔS

$$\ln K_{T_2} = \ln K_{T_1} + \frac{-\left(\Delta H^o_{T_2} - T_2\Delta S^o_{T_2}\right)}{RT_2} - \frac{-\left(\Delta H^o_{T_1} - T_1\Delta S^o_{T_1}\right)}{RT_1}$$

$$\ln K_{T_2} = \ln K_{T_1} + \frac{\left(-\Delta H^o_{T_2} + T_2\Delta S^o_{T_2}\right)}{RT_2} + \frac{\left(\Delta H^o_{T_1} - T_1\Delta S^o_{T_1}\right)}{RT_1}$$

$$\ln K_{T_2} = \ln K_{T_1} + \frac{\Delta H^o_{T_1}}{RT_1} - \frac{\Delta H^o_{T_2}}{RT_2} + \frac{\Delta S^o_{T_2} - \Delta S^o_{T_1}}{R}$$

If we are trying to expand this over a temperature range which is narrow enough that we can assume ΔH and ΔS are temperature independent, then we can simplify this to:

$$\ln K_{T_2} = \ln K_{T_1} + \frac{\Delta H^o_T}{R}\left(\frac{1}{T_1} - \frac{1}{T_2}\right) = \ln K_{T_1} + \frac{\Delta H^o_T}{R}\left(\frac{T_2 - T_1}{T_1 T_2}\right)$$

If $\Delta H<0$ (exothermic reaction) then increasing temperature lowers K, pushing the reaction towards reactants. This is precisely what your intuition should tell you (*think Le Chatelier*). The opposite is true for endothermic reactions.

Alternatively, we can come up with a more general expression. Remembering our

yields

$$\left(\frac{\partial (G/T)}{\partial T}\right)_P = \frac{1}{T}\left(\frac{\partial H}{\partial T}\right)_P - \frac{1}{T^2}H - \left(\frac{\partial S}{\partial T}\right)_P$$

Finally, remember that *at equilibrium* $\Delta C_p^o = \left(\frac{\partial \Delta H^o}{\partial T}\right)_p = T\left(\frac{\partial \Delta S^o}{\partial T}\right)_p$

$$\frac{\partial \left(\frac{\Delta G^o}{T}\right)}{\partial T} = \frac{1}{T}\Delta C_p^o - \frac{\Delta H^o}{T^2} - \frac{1}{T}\Delta C_p^o$$

$$\left(\frac{\partial \left(\Delta G^o / T\right)}{\partial T}\right)_p = \frac{-\Delta H^o}{T^2} \quad \text{(Gibbs-Helmholtz equation)}$$

The integrated form of this expression is:

$$\int_{T_1}^{T_2} \partial\left(G/T\right) = \int_{T_1}^{T_2} \frac{-H}{T^2}\,\partial T$$

$$\frac{\Delta G_{T_2}}{T_2} - \frac{\Delta G_{T_1}}{T_1} = -\int_{T_1}^{T_2} \frac{\Delta H_T}{T^2}\,\partial T$$

Now, if ΔH is independent of temperature, this will eventually lead to the expression above for the temperature dependence of ln(K) - try it!

For the unfolding of a protein, we can assume a simple "two-state" model (in other words, the protein is either completely folded or "completely" unfolded, but intermediates do not accumulate significantly). You might think that this is an unreasonable assumption, however the folding of a protein is a highly cooperative process, and one that is often poised on the edge of stability, such that quite often we can safely make this assumption.

The following represent thermodynamic data for the unfolding of ribonuclease at 30°C. In other words, for the reaction: F <--> U

Note the large ΔC_p^o.

pH	ΔG^o (kcal/mole)	ΔH^o (kcal/mole)	$T\Delta S^o$ (kcal/mole)	ΔS^o (cal deg^{-1} mole^{-1})	ΔC_p^o (cal deg^{-1} mole^{-1})
1.13	*-1.09*	60.3	61.2	202	2072
2.50	0.91	57.2	56.3	186	1985
3.15	3.09	53.0	50.0	165	1987

Note the large ΔC_p^o. For comparison, heat capacities for pure liquids are:

water: 18 cal deg^{-1} mole^{-1} benzene: 32 cal deg^{-1} mole^{-1}

Note that the protein is unstable at pH=1.13, but becomes stable at pH=2.50. Note also that ΔH^o and ΔS^o are large relative to ΔG^o. The large ΔC_p^o indicates that ΔH^o and ΔS^o will show a strong temperature dependence.

The following data represent thermodynamic parameters for the unfolding of a number of different proteins. Again, note the large ΔC_p^o.

Protein	ΔG^o (kcal/mole)	ΔH^o (kcal/mole)	$T\Delta S^o$ (kcal/mole)	ΔS^o (cal deg^{-1} mole^{-1})	ΔC_p^o (cal deg^{-1} mole^{-1})	$T_{max\ stabil}$ (°C)
Ribonuclease (pH 2.5, 30°C)	0.9	57	56.1	185	2000	-9
Chymotrypsin (pH 3, 25°C)	7.3	39	31.3	105	2600	10

Myoglobin (pH 9, 25°C)	13.6	42	28	95	1400	<0
β-lactoglobulin (5 M urea, pH 3, 25°C)	0.6	-21	-21.6	-72	2150	35

Ligand Binding and Related Equilibria

The simplest ligand binding reaction can be written: P + L ↔ PL with $K = \frac{[PL]}{[P][L]}$

$$\bar{v} = \frac{\text{\# moles ligand bound}}{\text{\# moles protein}} = \frac{[L_B]}{[P]+[PL]} = \frac{[L_B]}{[P_T]} = \frac{[PL]}{[P]+[PL]}$$

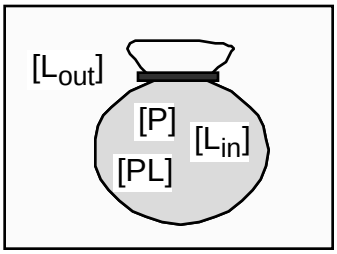
For the simple case described above, it will range from 0 to 1.

Methods of measurement

Equilibrium Dialysis:

For the binding of a small molecule (ligand, L) to a large macromolecule (protein, P), we can often measure binding via a technique called equilibrium dialysis. The requirements are:

1) a semiporous dialysis membrane to which L is freely permeable and P is not
2) a method for detecting $[L_{out}]$ and/or $[L_{in}]$ - a radioactive tag on L is most commonly used.



Perturbation of Ligand or Protein

Alternatively, if we can measure a perturbation which occurs on binding, we can readily measure the *fraction saturation* $\theta = \frac{\bar{v}}{n} = \frac{\Delta Obs}{\Delta Obs_T}$

This measurement *assumes* $\bar{v}$ is linear in *Obs* and is the same for different binding sites. Note that for n=1, $\bar{v} = \theta$.

Theoretical Treatment

Single Site per Macromolecule **(n=1)**

$$P + L \leftrightarrow PL \qquad K_a = \frac{[PL]}{[P][L]} \qquad K_d = \frac{[P][L]}{[PL]} = \frac{1}{K_a}$$

The equilibrium can be expressed either as an association (K_a) or a dissociation (K_d).

$$\bar{v} = \frac{[PL]}{[P]+[PL]} = \frac{1}{\left([P]/[PL]\right)+1} = \frac{1}{\left([P]/K_a[P][L]\right)+1}$$

$$\bar{v} = \frac{K_a[L]}{1+K_a[L]} = \frac{[L]}{1/K_a+[L]} = \frac{[L]}{K_d+[L]}$$

Always check the behavior of your equation at simple, extreme limits:

Various results: as $[L] \rightarrow \infty$, $\bar{v} \rightarrow 1$ (makes sense)
also when $\bar{v} = 0.5$, $[L] = 1/K_a = K_d$.

Rearrange to linear function (but watch for distortion of error)

$$\bar{v} = \frac{K_a[L]}{1+K_a[L]} = \frac{[L]}{\left(1/K_a\right)+[L]} \quad \text{then} \quad \left(1/K_a\right)+[L] = \frac{[L]}{\bar{v}}$$

$$\left(1/[L]K_a\right) = \frac{1}{\bar{v}} - 1 = \frac{1-\bar{v}}{\bar{v}} \quad \text{and} \quad \frac{\bar{v}}{[L]} = K_a(1-\bar{v})$$

The last form of the equation is known as a **Scatchard Plot**.

Alternatively, $\left(1/[L]K_a\right) = \frac{1-\bar{v}}{\bar{v}}$ leads to $\frac{\bar{v}}{1-\bar{v}} = [L]K_a$

Multiple Binding Sites

If we assume, more than one ligand binding site per protein, we have the more general form:

$$\bar{v} = \frac{[L_B]}{[P]+[PL]} = \frac{[L_B]}{[P_T]} = \frac{[PL]+2[PL_2]+\ldots+n[PL_n]}{[P]+[PL]+[PL_2]+\ldots+[PL_n]}$$

It should be obvious that as we approach saturation in this case, all protein is pushed to the $[PL_n]$ limit, and this ratio should approach *n*, the total number of binding sites per protein.

In equilibrium dialysis, if we know the concentration of protein and can measure the total concentration of ligand inside the bag (radioactive labeling is frequently employed), then we can measure this parameter.

$P + nL \leftrightarrow PL_n$ multiple sites, could be same or different, independent or cooperative.

Digression - Microscopic vs. Macroscopic

Examine the titration of glycine:

$^{+}H_3NCH_2COO\mathbf{H} \rightleftharpoons {}^{+}H_3NCH_2COO^- + H^+ \quad k_1$

$^{+}\mathbf{H}_3NCH_2COOH \rightleftharpoons H_2NCH_2COOH + H^+ \quad k_2$

$^{+}\mathbf{H}_3NCH_2COO^- \rightleftharpoons H_2NCH_2COO^- + H^+ \quad k_3$

$H_2NCH_2COO\mathbf{H} \rightleftharpoons H_2NCH_2COO^- + H^+ \quad k_4$

The reactions above represent *microscopic* equilibria (hence the lower case k).

More commonly we would measure the macroscopic equilibria:

$G\text{-}H2^+ \rightleftharpoons G\text{-}H + H^+ \quad K1$

$G\text{-}H \rightleftharpoons G^- + H^+ \quad K2$

where

$G\text{-}H_2^+ = {}^{+}H_3NCH_2COOH$

$GH = {}^{+}H_3NCH_2COO^- + H_2NCH_2COOH$

$G^- = H_2NCH_2COO^-$

then

$K_1 = k_1 + k_2$ and $K_2 = \dfrac{1}{\left(1/k_3 + 1/k_4\right)}$ (try it!)

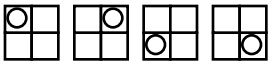
Note also that all four microscopic constants k_1, k_2, k_3, and k_4 are *not* independent.

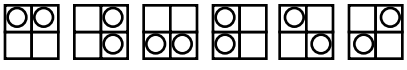
Ligand Binding - Identical Independent Sites

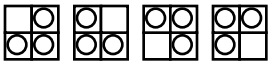
In general, one can arrange i ligands on n identical sites $\Omega_{n,i} = \dfrac{n!}{(n-i)!\,i!}$ ways

As an example, let's consider four independent sites (n=4)

$M_o =$  $\Omega_{n,i} = \dfrac{4!}{4!\,0!} = 1$

$M_o + L \rightleftharpoons M_1$ $M_1 =$  $\Omega_{n,i} = \dfrac{4!}{3!\,1!} = 4$

$M_1 + L \rightleftharpoons M_2$ $M_2 =$  $\Omega_{n,i} = \dfrac{4!}{2!\,2!} = 6$

$M_2 + L \rightleftharpoons M_3$ $M_3 =$  $\Omega_{n,i} = \dfrac{4!}{1!\,3!} = 4$

$M_3 + L \rightleftharpoons M_4$ $M_4 =$  $\Omega_{n,i} = \dfrac{4!}{0!\,4!} = 1$

As before, we want to calculate the macroscopic parameter

$$\bar{v} = \frac{[L_B]}{[P]+[PL]} = \frac{[L_B]}{[P_T]} = \frac{[PL]+2[PL_2]+\ldots+n[PL_n]}{[P]+[PL]+[PL_2]+\ldots+[PL_n]}$$

more generally

$$\bar{v} = \frac{\sum_{i=0}^{n} iM_i}{\sum_{i=0}^{n} M_i} = \frac{\sum_{i=0}^{n} i[PL_i]}{\sum_{i=0}^{n} [PL_i]}$$

We have the *macroscopic* equilibrium constants: $K_i = \dfrac{[M_i]}{[M_{i-1}][L]}$

or $[M_i] = K_i [L][M_{i-1}]$

Since these are identical sites, $k_i = k_j$, however, $K_i \neq K_j$

You can show that $[M_i] = [L]^i [M_o] \prod_{j=1}^{i} K_j$ (try it, solve for M_1, then for M_2, etc.)

It can be shown that $K_i = \frac{\Omega_{n,i}}{\Omega_{n,i-1}} k$ (in above example, $\Omega_{n,i}$ is the number of microstates)

as an aside, in example above $K_1=4k$, $K_2=(6/4)K$, $K_3=(4/6)k$, $K_4=(1/4)k$

This says that equilibrium favors adding a ligand to a fully unbound form is more favorable than adding ligand to a partially bound form. Does this make qualitative sense from your understanding so far?

then $K_i = \frac{\Omega_{n,i}}{\Omega_{n,i-1}} k = \frac{(n-i+1)!(i-1)!}{n!} \frac{n!}{(n-i)!i!} k = \frac{(n-i+1)}{i} k$

So that $[M_i] = [L]^i [M_o] \prod_{j=1}^{i} \left(\frac{(n-j+1)}{j} \right) k$

and finally $[M_i] = \left[\prod_{j=1}^{i} \left(\frac{(n-j+1)}{j} \right) \right] (k[L])^i [M_o]$

which simplifies somewhat to $[M_i] = \left[\frac{n!}{(n-i)!i!} \right] (k[L])^i [M_o]$

Returning to the macroscopic observable:

$$\bar{\nu} = \frac{\sum_{i=0}^{n} i M_i}{\sum_{i=0}^{n} M_i} = \frac{\sum_{i=0}^{n} \left\{ i \left[\frac{n!}{(n-i)!i!} \right] (k[L])^i [M_o] \right\}}{\sum_{i=0}^{n} \left\{ \left[\frac{n!}{(n-i)!i!} \right] (k[L])^i [M_o] \right\}} = \frac{[M_o] \sum_{i=0}^{n} \left\{ i \left[\frac{n!}{(n-i)!i!} \right] (k[L])^i \right\}}{[M_o] \sum_{i=0}^{n} \left\{ \left[\frac{n!}{(n-i)!i!} \right] (k[L])^i \right\}}$$

$$= \frac{0 + \sum_{i=1}^{n} \left\{ i \left[\frac{n!}{(n-i)!i!} \right] (k[L])^i \right\}}{1 + \sum_{i=1}^{n} \left\{ \left[\frac{n!}{(n-i)!i!} \right] (k[L])^i \right\}}$$

However, the denominator is a binomial expansion:

$$1 + \sum_{i=1}^{n} \left\{ \left[\frac{n!}{(n-i)!i!} \right] (k[L])^i \right\} = (1 + k[L])^n$$

eg. for n=4 above, $1 + \sum_{i=1}^{4} \left\{ \left[\frac{4!}{(4-i)!i!} \right] (k[L])^i \right\} = 1 + 4kL + 6k^2L^2 + 4k^3L^3 + k^4L^4 = (1 + k[L])^4$

save this, we'll need it. But in the meantime, take it and

differentiate both sides with $\frac{\partial}{\partial (k[L])}$ $\quad \sum_{i=1}^{n} \left\{ i \left[\frac{n!}{(n-i)!i!} \right] (k[L])^{i-1} \right\} = n(1 + k[L])^{n-1}$

then multiply both sides by k[L] $\quad \sum_{i=1}^{n} \left\{ i \left[\frac{n!}{(n-i)!i!} \right] (k[L])^i \right\} = nk[L](1 + k[L])^{n-1}$

Combining the above two results,

$$\bar{\nu} = \frac{nk[L](1 + k[L])^{n-1}}{(1 + k[L])^n} = \frac{nk[L]}{1 + k[L]}$$

Compare:

Before *for a single site*: $\bar{\nu} = \frac{K_a[L]}{1+K_a[L]}$, which led to $\frac{\bar{\nu}}{[L]} = K_a(1-\bar{\nu})$ and $\frac{\bar{\nu}}{1-\bar{\nu}} = [L]K_a$

Before, as [L]→∞, $\bar{\nu} \to 1$ (single site)
Now, as [L]→∞, $\bar{\nu} \to n$ (n multiple sites)
Both of these results should be completely and simply obvious. At complete binding, the number of ligands bound per protein is simply the number of ligand binding sites per protein.

Now, similarly for multiple, independent sites we have $\frac{\bar{\nu}}{[L]} = k(n-\bar{\nu})$

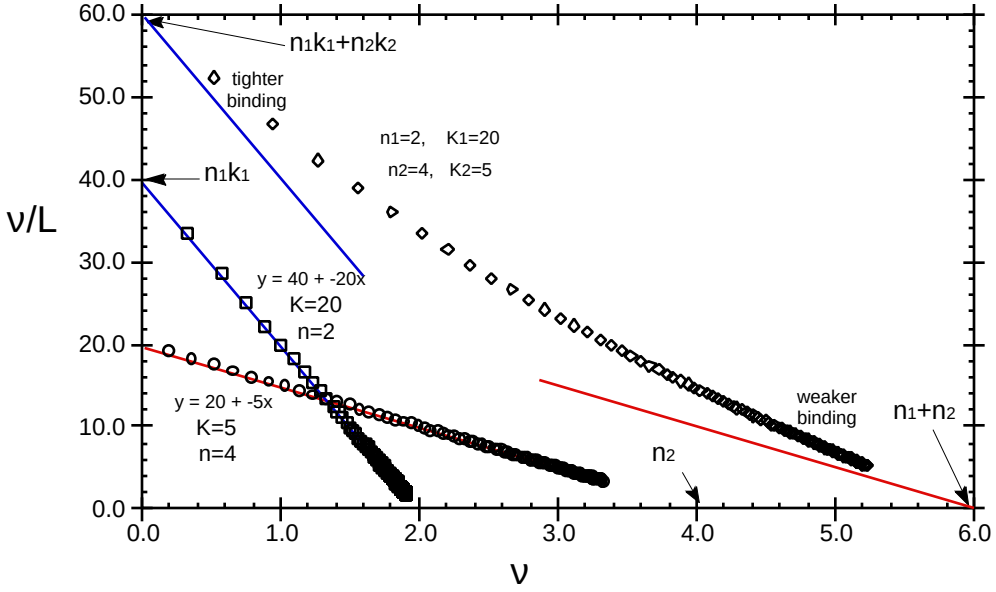
Ligand Binding - Multiple (Different) Classes of Independent Sites

If a protein has multiple class of sites in which sites within a given class are identical and independent, but there is more than one such class of sites, then for *m* different classes of sites, in which each class is composed of n_i identical sites with association constant k_i:

$$\bar{\nu} = \sum_{i=1}^{m} \frac{n_i k_i [L]}{1+k_i[L]} \quad \text{or} \quad \frac{\bar{\nu}}{[L]} = \sum_{i=1}^{m} \frac{n_i k_i}{1+k_i[L]} \quad \text{(Scatchard)}$$

Notice that if we plot $\frac{\bar{\nu}}{[L]}$ vs. $\bar{\nu}$, then the intercept ($\bar{\nu}$=0 at [L]=0) corresponds to $\frac{\bar{\nu}}{[L]} = \sum_{i=1}^{n} n_i k_i$

Similarly, the x-intercept ($\frac{\bar{\nu}}{[L]} = 0$) corresponds to $\bar{\nu} = n_1 + n_2$ (extra credit for the first to derive it!).



Ligand Binding - Multiple Identical but Interdependent Sites

If we assume a protein (or other macromolecule) with multiple ligand binding sites, for which binding of each ligand alters the energetics of binding of the subsequent ligand.
If the inherent (independent) binding constant is k_o, and we can express the change in free energy associated with increasing fractional binding as RTφ(ν),

then $\Delta G^o = \Delta G_o^o + RT\phi_\nu$ where $\Delta G_o^o = -RT\ln k_o$

and then $k(\nu) = e^{-\Delta G^o/RT} = e^{-\left(\Delta G_o^o + RT\phi_\nu\right)/RT} = e^{-\Delta G_o^o/RT} e^{-\phi_\nu} = k_o e^{-\phi_\nu}$

Stop and analyze:
As ligand binds, if:

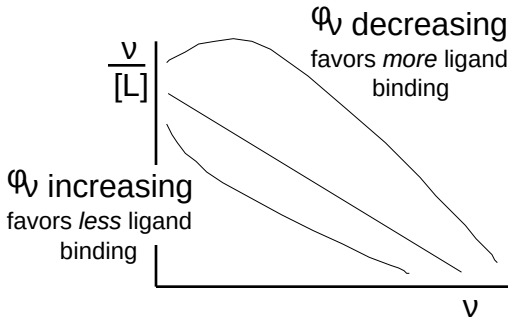
$\Delta\phi_\nu < 0$ then $k > k_o$	tighter binding cooperative
$\Delta\phi_\nu > 0$ then $k < k_o$	weaker bindinganticooperative

One can then replace k in the multiple independent sites model:

$$\bar{\nu} = \frac{nk[L]}{1+k[L]} = \frac{n[L]k_o e^{-\phi_\nu}}{1+[L]k_o e^{-\phi_\nu}} = \frac{n[L]k_o}{e^{\phi_\nu}+[L]k_o}$$

$$\frac{\bar{\nu}}{[L]} = k(n-\bar{\nu}) = (n-\bar{\nu})k_o e^{-\phi_\nu}$$

The plot at right shows behavior characterized by these kinds of interactions. (Note that our convention for k is the inverse of that used by Cantor & Schimmel, such that a decreasing φ_ν corresponds to a decreasing energy cost of *association*, and consequently more ligand is bound than in the independent site model. Conversely, an increasing φ_ν indicates a larger barrier to binding of second, third, and fourth ligands, so that less is bound at any point in the titration.



N.B. - the behavior predicted for φ_ν increasing (anticooperative binding) is similar in general shape to that predicted for multiple classes of independent sites. In practice, it may be hard to distinguish the two. C & S suggests that multiple classes of independent sites is more simple. ***One should always choose the most simple model which satisfactorily explains the data.***

In contrast, the cooperative behavior predicted for φ_ν decreasing cannot be explained by a simpler model.

Finally, one can (with great caution in real experimental situations) apply the same type of reasoning to a system with multiple class of interacting sites, each class having a different interaction function φ_ν.

N.B. - It is less important that you memorize these different equations, and more important that you understand them and can derive them. It may well be that a system you encounter in the future requires a slight twist from these stock solutions - you want to be able to come up with a correct, custom solution.

Ligand Binding - Infinite Cooperativity

If binding to multiple sites is infinitely cooperative, then binding is all or none and can be written quite simply as:

$$M_o + nL \leftrightarrows M_n \qquad \text{then} \qquad K_a^n = \frac{[M_n]}{[M_o][L]^n}$$

we can derive ν as before, $\nu = \dfrac{n[M_n]}{[M_o]+[M_n]} = \dfrac{nK_a^n[M_o][L]^n}{[M_o]+K_a^n[M_o][L]^n} = \dfrac{nK_a^n[L]^n}{1+K_a^n[L]^n}$

$$\nu = \frac{n[L]^n}{\left(\frac{1}{K_a^n}\right)+[L]^n} \quad \text{and} \quad \nu = \frac{nK_a^n[L]^n}{1+K_a^n[L]^n} \quad \text{or trivially} \quad \frac{\nu}{[L]} = \frac{nK_a^n[L]^{n-1}}{1+K_a^n[L]^n}$$

bringing $\left(\frac{1}{K_a^n [L]^n}\right) + 1 = \frac{n}{\nu}$

from here we can go to $\frac{\nu}{n} = \theta = \frac{K_a^n [L]^n}{K_a^n [L]^n + 1}$ ← Another useful form.

Note that $\frac{\theta}{1-\theta} = \frac{\frac{K_a^n [L]^n}{K_a^n [L]^n + 1}}{1 - \frac{K_a^n [L]^n}{K_a^n [L]^n + 1}} = \frac{K_a^n [L]^n}{K_a^n [L]^n + 1 - K_a^n [L]^n} = K_a^n [L]^n$

So that $\ln\left(\frac{\theta}{1-\theta}\right) = \ln\left(K_a^n [L]^n\right) = n \ln\left(K_a [L]\right) = n \ln K_a + n \ln[L]$

and plotting $\ln\left(\frac{\theta}{1-\theta}\right)$ vs. $\ln[L]$ is predicted to yield a straight line with slope of n and an intercept of n ln K.

You will also see this in other forms. Note that $\frac{\theta}{1-\theta} = \frac{\nu}{n-\nu}$ (easy, try it!)

Ligand Binding - Intermediate Cooperativity

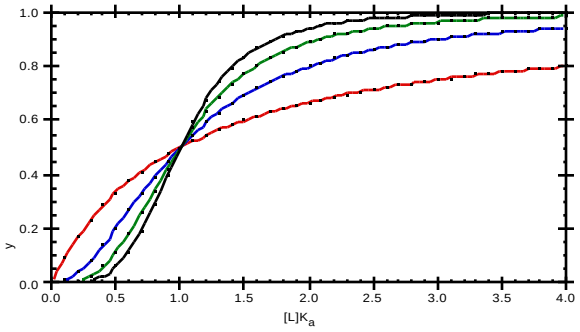
A *semiemperical* derivative of this which is often used is $\frac{\nu}{[L]} = \frac{n K_a^{\alpha_H} [L]^{\alpha_H - 1}}{1 + K_a^{\alpha_H} [L]^{\alpha_H}}$

In this case α_H is called the Hill constant and indicates the degree of cooperativity. As α_H approaches n, then the binding is infinitely cooperative.

Other manipulations of these equations are:

$\theta = \frac{\nu}{n} = \frac{K_a^n [L]^n}{1 + K_a^n [L]^n} \rightarrow \frac{K_a^{\alpha_H} [L]^{\alpha_H}}{1 + K_a^{\alpha_H} [L]^{\alpha_H}} = \frac{\left(K_a [L]\right)^{\alpha_H}}{1 + \left(K_a [L]\right)^{\alpha_H}}$

The figure below plots θ vs. K[L] from the above equation and demonstrates it's behavior for infinite cooperativity for α_H = n = 1,2,3,4.



Note that n = 1 is a single site (of course, uncooperative) and shows a typical titration profile. In the other cases, ligand binds slowly at first, but as a few bind, more bind with little (no) energetic cost.

NOTE - α_H is not a constant in this treatment - see below.

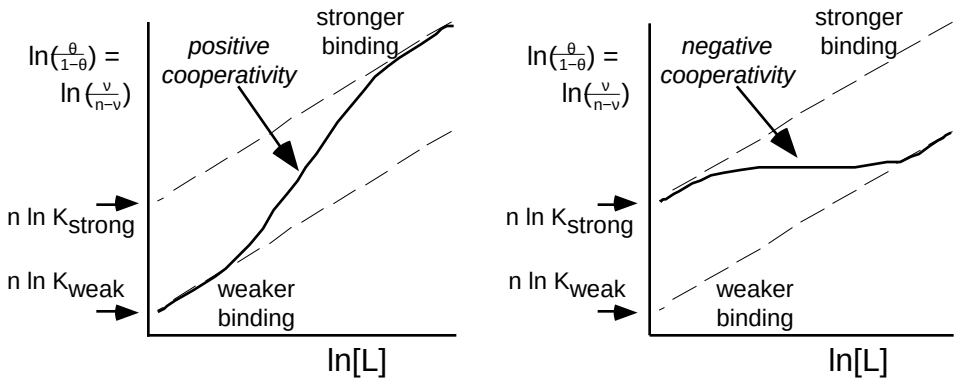
Hill Plots

Also, as before we have $\ln\left(\frac{\theta}{1-\theta}\right) = \alpha_H \ln K_a + \alpha_H \ln[L]$

A plot of $\ln\left(\frac{\theta}{1-\theta}\right)$ vs. ln[L] yields a (varying) slope of αH (these are usually called Hill Plots)

For the plot above (infinite cooperativity), this manipulation would yield a straight line with slope = α_H = n. Again, for **no** cooperativity, the slope would be 1.0.

Now, what happens when we have intermediate cooperativity?



For positive cooperativity, binding is "weak" early in the titration when no sites are occupied, and so the sites titrate as if there is no cooperativity (in this region α_H=1). Very late in the titration, as you are filling the last remaining sites (now with a strong binding constant), you again see little evidence of cooperativity (in this region, again α_H=1).. In the middle, you see the largest evidence of cooperativity (α_H>1).

For negative cooperativity, binding starts off at the "strong" limit, and then gets weaker as more sites are filled.

The plots above are usually called "Hill Plots." Note that you will see different nomenclature for the same plot. As before, the limiting conditions have α_H=1, while in the middle of the titration α_H<1. The Hill constant is usually defined as the slope of the curve at the midpoint of the titration. In other words, where cooperativity (or anticooperativity) is *greatest*.

Review - Ligand Binding

NOTE: van Holde uses K_a as we do, however, Cantor & Schimmel use K_d (= $1/K_a$). Be warned. Similarly, van Holde uses n_α instead of α_H (as we and Cantor & Schimmel do...).

Terms:

$\bar{\nu} = \frac{[L_B]}{[P_T]}$ = $\frac{\text{conc of ligand bound}}{\text{total concentration of protein}}$ can range from 0 to n (number of binding sites/protein)

fraction saturation $\theta = \frac{\bar{\nu}}{n} = \frac{\Delta Obs}{\Delta Obs_T}$ can range from 0 to 1

Single Site

$\frac{\bar{\nu}}{[L]} = \frac{K_a}{1 + K_a[L]} = -K_a\bar{\nu} + K_a$ Plot $\frac{\bar{\nu}}{[L]}$ vs. $\bar{\nu}$, slope = -*K*, y-intercept = *K*

Plot $\frac{\bar{\nu}}{[L]}$ vs. $[L]$, y-intercept = *K*

Multiple Identical, Independent Sites

$\frac{\bar{\nu}}{[L]} = \frac{nK_a}{1 + K_a[L]} = -K_a\bar{\nu} + nK_a$ Plot $\frac{\bar{\nu}}{[L]}$ vs. $\bar{\nu}$, slope = -*K*, y-intercept = n*K*

Plot $\frac{\bar{\nu}}{[L]}$ vs. $[L]$, y-intercept = *K*

Multiple Independent *Classes* of Multiple Identical, Independent Sites

$\frac{\bar{\nu}}{[L]} = \sum_{i=1}^{n_{classes}} \frac{n_i K_{a_i}}{1 + K_{a_i}[L]}$ Plot $\frac{\bar{\nu}}{[L]}$ vs. $[L]$, y-intercept = $\sum_{i=1}^{n_{classes}} n_i K_{a_i}$

Multiple Identical, Infinitely Cooperative Sites

$\frac{\bar{\nu}}{[L]} = \frac{nK_a^n[L]^{n-1}}{1 + K_a^n[L]^n}$ $\theta = \frac{\bar{\nu}}{n} = \frac{K_a^n[L]^n}{1 + K_a^n[L]^n}$

then

$\ln\left(\frac{\theta}{1-\theta}\right) = n \ln K_a + n \ln[L]$ Plot $\ln\left(\frac{\theta}{1-\theta}\right)$ vs. $\ln[L]$, slope = *n*, y-intercept = $n\ln K_a$

Multiple Identical Sites, Intermediate Cooperativity

$\frac{\bar{\nu}}{[L]} = \frac{nK_a^{\alpha_H}[L]^{\alpha_H - 1}}{1 + K_a^{\alpha_H}[L]^{\alpha_H}}$ $\theta = \frac{\bar{\nu}}{n} = \frac{K_a^{\alpha_H}[L]^{\alpha_H}}{1 + K_a^{\alpha_H}[L]^{\alpha_H}}$

then

$\ln\left(\frac{\theta}{1-\theta}\right) = \alpha_H \ln K_a + \alpha_H \ln[L]$ Plot $\ln\left(\frac{\theta}{1-\theta}\right)$ vs. $\ln[L]$, slope = α_H y-intercept = $\alpha_H \ln K_a$

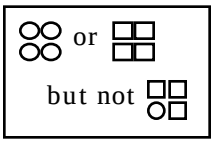
For positive cooperativity, $1 \le \alpha_H \le n$ (Fully Independent ≤ ≤ Infinitely Cooperative)

Models for Cooperativity

MWC - Monod, Wyman, Changeux

Assume that each subunit of a multisubunit protein can exist in two states:
- ○ R (stronger ligand binding) and
- □ T (weaker ligand binding).

Assume that all subunits are symmetric and that they can be either all R or all T, but not intermediate (you can argue that this *should* follow from the symmetry of the system).



In the absence of ligand, an equilibrium exists:

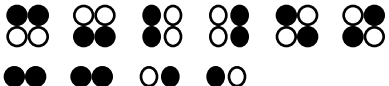
$$R \leftrightharpoons T \qquad \text{w / equilib const } L = \frac{T}{R}$$

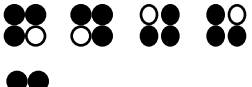
Le Chatelier tells us that addition of ligand will shift the equilibrium to the left (L will decrease).

Backing up to examine the R and T states separately, each can be considered a system with n identical sites and we can look at the microscopic equilibrium constants (k_R and k_T) for each.

Let's look at a system with four sites (hemoglobin?). Using nomenclature similar to above, the microscopic states are given by:

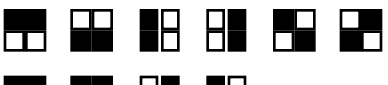
$r_o + L \rightleftharpoons r_1$ 

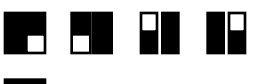
$r_1 + L \rightleftharpoons r_2$ 

$r_2 + L \rightleftharpoons r_3$ 

$r_3 + L \rightleftharpoons r_4$ 

$t_o + L \rightleftharpoons t_1$ 

$t_1 + L \rightleftharpoons t_2$ 

$t_2 + L \rightleftharpoons t_3$ 

$t_3 + L \rightleftharpoons t_4$ 

$$k_r = \frac{[r_1]}{[r_o][L]} = \frac{[r_2]}{[r_1][L]} = \frac{[r_3]}{[r_2][L]} = \frac{[r_4]}{[r_3][L]} \qquad k_t = \frac{[t_1]}{[t_o][L]} = \frac{[t_2]}{[t_1][L]} = \frac{[t_3]}{[t_2][L]} = \frac{[t_4]}{[t_3][L]}$$

The macroscopic equivalents are:

$R_o + L \rightleftharpoons R_1$ $\qquad$ $T_o + L \rightleftharpoons T_1$

$R_1 + L \rightleftharpoons R_2$ $\qquad$ $T_1 + L \rightleftharpoons T_2$

$R_2 + L \rightleftharpoons R_3$ $\qquad$ $T_2 + L \rightleftharpoons T_3$

$R_3 + L \rightleftharpoons R_4$ $\qquad$ $T_3 + L \rightleftharpoons T_4$

$$K_{R_i} = \frac{[R_i]}{[R_{i-1}][L]} = \frac{\Omega_{n,i}}{\Omega_{n,i-1}} k_r \qquad K_{T_i} = \frac{[T_i]}{[T_{i-1}][L]} = \frac{\Omega_{n,i}}{\Omega_{n,i-1}} k_t$$

remember $\Omega_{n,i} = \frac{n!}{(n-i)!\,i!}$

so $K_{R_i} = \frac{[R_i]}{[R_{i-1}][L]} = \frac{\Omega_{n,i}}{\Omega_{n,i-1}} k_r = \frac{(n-i+1)!(i-1)!}{n!} \frac{n!}{(n-i)!\,i!} k_r = \frac{(n-i+1)}{i} k_r$

and $K_{T_i} = \frac{[T_i]}{[T_{i-1}][L]} = \frac{(n-i+1)}{i} k_t$

so that for the above, $K_{R1} = 4k_r$, $K_{R2} = (3/2)k_r$, $K_{R3} = (2/3)k_r$, $K_{R4} = (1/4)k_r$ etc...

Again, think about this result. Does it make sense?

and ultimately (just as before): $T_i = \left[\frac{n!}{(n-i)!\,i!}\right] k_t^i L^i T_o$ and $R_i = \left[\frac{n!}{(n-i)!\,i!}\right] k_r^i L^i R_o$

From the definition of $\bar{\nu}$, we have $\bar{\nu} = \frac{\text{total L bound}}{\text{total \# sites}} = \frac{\sum_{i=1}^{n} iR_i + \sum_{i=1}^{n} iT_i}{\sum_{i=1}^{n} R_i + \sum_{i=1}^{n} T_i}$

$$\bar{\nu} = \frac{\sum_{i=1}^{n} i\left[\frac{n!}{(n-i)!\,i!}\right] k_r^i L^i R_o + \sum_{i=1}^{n} i\left[\frac{n!}{(n-i)!\,i!}\right] k_t^i L^i T_o}{\sum_{i=1}^{n} \left[\frac{n!}{(n-i)!\,i!}\right] k_r^i L^i R_o + \sum_{i=1}^{n} \left[\frac{n!}{(n-i)!\,i!}\right] k_t^i L^i T_o} = \frac{R_o \sum_{i=1}^{n} i\left[\frac{n!}{(n-i)!\,i!}\right] k_r^i L^i + T_o \sum_{i=1}^{n} i\left[\frac{n!}{(n-i)!\,i!}\right] k_t^i L^i}{R_o \sum_{i=1}^{n} \left[\frac{n!}{(n-i)!\,i!}\right] k_r^i L^i + T_o \sum_{i=1}^{n} \left[\frac{n!}{(n-i)!\,i!}\right] k_t^i L^i}$$

we know that the free forms T_o and R_o are related by the equilib constant L, that is $T_o = L_{RT} R_o$.
and playing the same manipulation that we did previously relating this to $\bar{\nu}$, we have

$$\bar{\nu} = \frac{n k_r L \left(1 + k_r L\right)^{n-1} + L_{RT} n k_t L \left(1 + k_t L\right)^{n-1}}{\left(1 + k_r L\right)^n + L_{RT} \left(1 + k_t L\right)^n}$$

now by convention let the ratio of k_t and k_r equal c $\qquad c = \frac{k_t}{k_r}$

$$\bar{\nu} = n k_r L \frac{\left(1 + k_r L\right)^{n-1} + c L_{RT} \left(1 + c k_r L\right)^{n-1}}{\left(1 + k_r L\right)^n + L_{RT} \left(1 + c k_r L\right)^n}$$

Rearranging this to the form for a Hill Plot, we have

$$\frac{\bar{\nu}}{n - \bar{\nu}} = k_r L \frac{1 + c L_{RT} \left(\frac{1 + c k_r L}{1 + k_r L}\right)^{n-1}}{1 + L_{RT} \left(\frac{1 + c k_r L}{1 + k_r L}\right)^{n-1}}$$

As is always a good idea, let's look at the limits:

As $L \to 0$ $\qquad \frac{\bar{\nu}}{n - \bar{\nu}} \to k_r L \frac{1 + c L_{RT}}{1 + L_{RT}}$

$$\ln \frac{\bar{\nu}}{n - \bar{\nu}} \to \ln L + \ln \left(k_r \frac{1 + c L_{RT}}{1 + L_{RT}} \right) \quad \text{(Hill plot)}$$

As $L \to \infty$ $\qquad \frac{\bar{\nu}}{n - \bar{\nu}} \to k_r L \frac{1 + c L_{RT} \left(\frac{c k_r}{k_r}\right)^{n-1}}{1 + L_{RT} \left(\frac{c k_r}{k_r}\right)^{n-1}} = k_r L \frac{1 + L_{RT} c^n}{1 + L_{RT} c^{n-1}}$

$$\ln \frac{\bar{\nu}}{n - \bar{\nu}} \to \ln L + \ln \left(k_r \frac{1 + L_{RT} c^n}{1 + L_{RT} c^{n-1}} \right) \quad \text{(Hill plot)}$$

On a Hill Plot,we see that we predict a straight line with a slope of 1 for each case.
Finally, again going to limits, if T is very highly favored in the absence of ligand then

As $L \to 0$ and $L_{RT} \to \infty$ then $\ln \frac{\bar{\nu}}{n - \bar{\nu}} \to \ln L + \ln\left(k_r c\right) = \ln L + \ln k_t$

As expected, **early** in the titration the system behaves just like a simple **T** state.
If we additionally assume that c is small ($k_r >> k_t$ ligand binding is much stronger to the R state), then

As $L \to \infty$ and $c << 1$ then $\ln \frac{\bar{\nu}}{n - \bar{\nu}} \to \ln L + \ln k_r$

As expected, **late** in the titration the system behaves just like a simple **R** state.

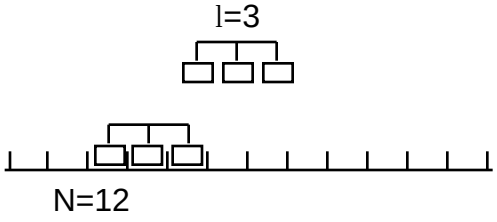
KNF - Koshland, Nemethy, Filmer

Although the MWC model does explain hemoglobin and various other systems very well, the MWC model makes the dramatic assumption that the protein can only exist all R or all T. This is most certainly not true for all systems.

The KNF model allows for mixed states of the subunits within a single protein and so is more general. It is also called the "sequential model" because subunit can sequentially (one at a time) convert from T to R and vice versa. It is also much more complicated mathematically and solutions depend on the exact form of the intersubunit energetics.

Lattice Site Binding

We can also examine a multi-site ligand binding to a multi-site lattice (the first examples that come to mind include proteins or drugs binding to DNA).



As before, we can take ν as the number of moles of L bound per mole of lattice.

In this case, N is the number of monomeric binding sites, however , N/*l* is the maximum number of multimeric ligands which can bind at saturation.. However, before

any ligand has bound, there are N-*l*+1 *potential* binding sites. To understand intermediate states, we must invoke statistical arguments as before.

$\nu = N_{fl} L k$ where N_{fl} is the average number of free ligand sites of length *l* per lattice and k is the intrinsic microscopic association constant, just as before.

Cantor & Schimmel derive (pp 878-881) the following expression

$$\frac{\nu}{L} = N\left(1 - \frac{l\nu}{N}\right) k \left[\frac{\left(1 - \frac{l\nu}{N}\right)}{1 - \frac{(l-1)\nu}{N}}\right]^{l-1}$$ there is the caveat that this is strictly only true for large N

As always, let's look at this in its limits:

For *l*=1 we have $\frac{\nu}{L} = N\left(1 - \frac{\nu}{N}\right) k \left[\frac{\left(1 - \frac{\nu}{N}\right)}{1 - \frac{(1-1)\nu}{N}}\right]^{1-1} = N\left(1 - \frac{\nu}{N}\right) k = Nk - \nu k$

Rearranging the general lattice equation we have $\frac{\nu}{L} = Nk\gamma - \nu k \frac{\gamma l}{N}$ where $\gamma = \left[\frac{\left(1 - \frac{l\nu}{N}\right)}{1 - \frac{(l-1)\nu}{N}}\right]^{l-1}$

For large *l* and small ν we have

$$\frac{\nu}{L} = Nk\gamma - \nu \frac{k}{N}\gamma l \quad \text{where} \quad \frac{k}{N}\gamma l = \frac{k}{N} l \left[\frac{\left(1 - \frac{l\nu}{N}\right)}{1 - \frac{(l-1)\nu}{N}}\right]^{l-1} \text{¾¾¾¾¾¾¾¾¾¾} \rightarrow l \frac{k}{N} (1)^{l-1} \text{¾¾¾¾} \rightarrow \infty$$

so that slope in a Scatchard plot goes to -∞, or more generally the slope gets more steep in a negative sense.

For large *l* and large ν (as ν approaches N/*l*), we have

$$\frac{\nu}{L} = Nk\gamma - \nu \frac{k}{N}\gamma l \quad \text{where} \quad \frac{k}{N}\gamma l = \frac{k}{N} l \left[\frac{\left(1 - \frac{l\nu}{N}\right)}{\left(1 - \frac{l\nu}{N}\right) - \frac{\nu}{N}}\right]^{l-1} = \frac{k}{N} l \left[\frac{0}{0 - \frac{\nu}{N}}\right]^{l-1} \text{¾¾¾¾} \rightarrow 0$$

So we see an initial steep negative slope which eventually levels out and approaches 0 as we are saturated.

Early in the titration, binding is strongly favored. This is a result of the increase in entropy of having ligand bound in many possible different sites. However, as the lattice becomes saturated there are fewer and fewer sites available and now entropy pushes against binding.

Kinetics of Ligand Binding / Reaction Kinetics

Unimolecular reactions (C&S Chapt. 16-2)

$X_0 \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} X_1$

The rate equation is $-\frac{dX_o}{dt} = k_1 X_o - k_{-1} X_1$

How might one follow such a reaction? One example is to perturb a system at equilibrium. An example is a temperature jump experiment. At the original temperature, the system is at equilibrium. If we "instantaneously" change the temperature to a new temperature, a new equilibrium distribution will be defined, but the system is sitting at the old distribution.

Take $\overline{X_o}$ as the new equilibrium concentration of X_o. Then

Kinetics of Protein Folding

Simple Two-State Unfolding

Kinetics

NOTE: Convention in kinetics is to use lower case *k* for kinetic constants and upper case *K* for equilibrium constants (except for microscopic equilibrium constants).

Important: Note that the stoichiometry of a reaction does not tell you anything about the kinetic mechanism of a reaction! This is very often overlooked.

Example:

For the reaction whose stoichiometry is: $aA + bB \rightarrow cC + dD$

The potential kinetic mechanisms could lead to almost any velocity equation, including:

$V = kA^a B^b$ or $V = kAB^2$ or $V = kA$ or $V = kB^2$ or $V = k$ or ...

Remember: the upper equation simply describes the *stoichiometry* of the reaction.

Why is this?

Because the underlying *kinetic* mechanism for the overall reaction

$$2A + 2B \rightarrow 2C$$

might be:

$V = kAB^2$ $\quad A + 2B \xrightarrow{\text{slow}} X \xrightarrow[+A,\ \text{fast}]{} Y \xrightarrow[\text{fast}]{+2B} 2C$

$V = kA$ $\quad A \xrightarrow{\text{slow}} A^* \xrightarrow[+A,\ \text{fast}]{} W \xrightarrow[\text{fast}]{+2B} 2C$

$V = kB^2$ $\quad 2B \xrightarrow{\text{slow}} V \xrightarrow[+A,\ \text{fast}]{} Z \xrightarrow[+A,\ \text{fast}]{} 2C$

and many other possibilities.

Simple kinetic mechanisms

Unimolecular, one-step reactions

e.g. protein folding $A \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} B$

$-\frac{dA}{dt} = k_1 A - k_{-1} B$ let $\Delta A = A - A_{eq}$ $\Delta B = B - B_{eq}$

then

$$-\frac{d\Delta A}{dt} = k_1\left(\Delta A + A_{eq}\right) - k_{-1}\left(\Delta B + B_{eq}\right)$$

note that from the stoichiometry, you know that $\Delta A = -\Delta B$

$$-\frac{d\Delta A}{dt} = k_1 \Delta A + k_1 A_{eq} + k_{-1}\Delta A - k_{-1} B_{eq} = \left(k_1 + k_{-1}\right)\Delta A + k_1 A_{eq} - k_{-1} B_{eq}$$

At equilibrium, $k_1 A_{eq} = k_{-1} B_{eq}$

$-\frac{d\Delta A}{dt} = \left(k_1 + k_{-1}\right)\Delta A$ which then leads simply to $-\int \frac{d\Delta A}{\Delta A} = \int \left(k_1 + k_{-1}\right) dt$

finally

$$-\ln \Delta A_t + \ln \Delta A_o = \ln \frac{\Delta A_o}{\Delta A_t} = (k_1 + k_{-1})(t - 0)$$

$$\Delta A_t = \Delta A_o e^{-(k_1 + k_{-1})t}$$

$$A_t = A_{eq} + (A_o - A_{eq}) e^{-(k_1 + k_{-1})t}$$

This tells us that A approaches exponentially its equilibrium value. Make sense?
A simple example of a unimolecular, one-step reaction is protein folding (see later).

Pre-equilibrium kinetics

(Leonor Michaelis and Maude Menten, 1913)

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} ES \xrightarrow{k_2} E + P$$

The simplest kinetic formalism falls directly from our equilibrium studies. If we assume that our reaction (k_2, above) is very slow relative to both the on (k_1) and off (k_{-1}) rates for binding of substrate to enzyme, then we can simply treat substrate binding as an equilibrium binding (using the equations above). In other words, the first step is always at equilibrium, unperturbed by the second step. The velocity of the reaction under the simplest of conditions is then $V = k_2[ES]$, where [ES] is determined as before for ligand binding (also remember that [ES] is proportional to $\bar{v}$, so that $V = k_2 K_a [E][S]$.
Solve for the equilibrium in terms of $[E]_t$ and $[S]_t$

$$K_a = \frac{[ES]}{[E][S]} \qquad [E]_t = [E] + [ES] \qquad [S]_t = [S] + [ES]$$

$$K_a = \frac{[ES]}{([E]_t - [ES])([S]_t - [ES])} = \frac{x}{(E_t - x)(S_t - x)}$$

$$K_a (E_t - x)(S_t - x) = x$$

This can be solved exactly very simply, but for simplicity, *if* we can assume substrate in excess, then:

$$K_a (E_t - x) S_t = x$$

$$K_a E_t S_t = K_a x S_t + x$$

$$x = [ES] = \frac{K_a E_t S_t}{K_a S_t + 1} = \frac{E_t S_t}{S_t + 1/K_a} = \frac{E_t S_t}{S_t + K_d}$$

Finally,

$$V = k_2[ES] = \frac{k_2 E_t S_t}{S_t + K_d}$$

Figuring that the reaction is fastest as $S_t \to \infty$, calculate V_{max} (try it!).
This looks very similar to the equation for simple ligand binding (as we would expect):

$$\bar{v} = \frac{K_a[L]}{1 + K_a[L]} = \frac{[L]}{1/K_a + [L]} = \frac{[L]}{K_d + [L]}$$

Steady state kinetics

(G. E. Briggs & J. B. S. Haldane, 1925)

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} ES \xrightarrow{k_2} E + P$$

If pre-equilibrium cannot be assumed, then we can take a slightly different approach
As before, we have the equations for mass conservation:

$$[E]_t = [E] + [ES] \qquad [S]_t = [S] + [ES]$$

We then *assume* that after the reaction has been running for some time, the rate for formation of [ES] equals the rate of its loss - we *assume* that [ES] has reached *steady state*:

$$-\frac{d[ES]}{dt} = 0 = k_1[E][S] - k_{-1}[ES] - k_2[ES]$$

$$(k_{-1} + k_2)[ES] = k_1[E][S]$$

$$\frac{k_1}{k_{-1} + k_2} = \frac{1}{K_m} = \frac{[ES]}{[E][S]}$$

We can see that this is just the same equation we dealt with above, except that $1/K_m$ substitutes for K_a (note that the latter is simply $1/K_d$).

It should be obvious that "pre-equilibrium" kinetics is simply a special case of the more general steady state kinetics (in pre-equilibrium kinetics, $k_{-1} >> k_2$).

The form of the solution for these reaction equations is the same as the form of the solution for the related binding equations. In the same way that we devised Scatchard and other plots for binding, we can do exactly the same for kinetic analyses. They now adopt new names.

Note that the same caveats apply as before, regarding the manipulation of raw data and the resulting effects on error analysis.

Similarly, the same kinds of analyses can be brought in to consider multiple sites, independent or dependent.

Finally, a reminder that the above analyses assume substrate in excess. For an enzymatic reaction, note that substrate is depleted with time, so that what is in excess initially may not be in excess near the end of the reaction. Also, since steady state is assumed, the equations are not valid for the very beginning of the reaction (pre-steady state kinetics).

Single Site Binding

$\frac{\bar{v}}{[L]} = \frac{K_a}{1 + K_a[L]} = -K_a\bar{v} + K_a$ Plot $\frac{\bar{v}}{[L]}$ vs. $\bar{v}$, slope = -*K*, y-intercept = *K* (Scatchard)

Single Site Kinetics

$$\frac{v}{[S]} = \frac{{}^{1}\!/\!_{K_m}\, k_2 E_t}{1 + {}^{1}\!/\!_{K_m}\,[L]} = \frac{{}^{1}\!/\!_{K_m}\, V_{\max}}{1 + {}^{1}\!/\!_{K_m}\,[L]}$$

$$\frac{v}{[S]} = -{}^{1}\!/\!_{K_m}\, v + {}^{V_{\max}}\!/\!_{K_m}$$

Plot $\frac{v}{[L]}$ **vs.** v, slope = $-1/K_m$, y-intercept = $Vmax/K_m$ (Eadie-Hofstee)

Exact solutions (numerical integration) of complex rate equations

The Folding of Proteins and Nucleic Acids

Quick review - the amino acids

Nonpolar - low solubility in water

Always: Ala, Val, Ile, Leu. Almost always: Phe, Trp, Met

Charged - high solubility in water, must interact strongly with water

Glu, Asp, Arg, Lys

Strongly Polar - need to interact with water

Asn, Gln, Ser, Thr

Ambiguous

Cys, His - pK_a's near 7.0. Clearly polar when charged, fairly nonpolar when neutral

Tyr - has a H-bond donor, but is otherwise very nonpolar. Indeed it is normally found in nonpolar niches in protein structures - rarely at the surface.

Special

Gly - nonpolar. More freedom to rotate than others (wider range of phi and psi angles).

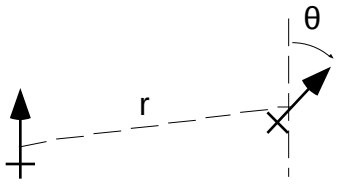
Pro - nonpolar. Less freedom to rotate than others (phi fixed, psi limited - see below).

The Forces that Govern Protein Folding

Coulombic Charge - Charge Interaction

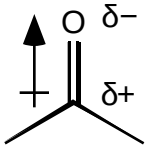
$$E_{coulombic} = \frac{1}{\varepsilon}\frac{q_1 q_2}{r^2}$$

Dipolar Interaction

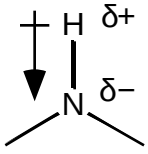


$$E_{dipolar} = \frac{1}{\varepsilon}\left[\frac{\vec{\mu}_A \cdot \vec{\mu}_B}{r^3} - \frac{3(\vec{\mu}_A \cdot \vec{r})(\vec{\mu}_B \cdot \vec{r})}{r^5}\right]$$

where μ_A and μ_B are point dipoles (vectors) separated by a distance r. Remember that carbonyl C=O and amide N-H bonds have an uneven distribution of electrons - that is, each has a dipole moment associated with it. Note from above that the dipolar interaction depends on both the *distance* and the *angle* between the dipoles.



Point charge approximation. One can model the dipolar interaction using a point dipole approximation as above, or alternatively, since the distance between dipoles is short, it may be better to view each dipole as two separated charges. This is shown in the lower figure at right. We can now view the interactions as simple coulombic charge-charge interactions, according to:



$$E_{dipolar} = \sum_{i,j}\frac{1}{\varepsilon}\frac{q_i q_j}{r_{ij}^2}$$

This is a common approach in "molecular dynamics/mechanics" calculations. Each atom in the entire protein is assigned a partial charge and the coulombic interactions are summed. Again note that an important parameter in this estimation is the dielectric (ε) of the intervening medium. Values from 2-5 or a bit higher are realistic. (ε for water is 80!). In this case, there is little between the charges and so a low dielectric is reasonable. For dipoles which are separated a greater distance in the protein, The effective dielectric of the intervening protein will be somewhat higher. However, estimates are difficult to make and the value will be different in different parts of the protein.

Dipole - Point Charge Interactions

You can readily imagine interactions between a dipole moment from one part of the protein structure and a point charge nearby (a charged amino acid for example). This intermediate case is again well approximated by the full point charge picture presented above.

van der Waals Interactions

this interaction is a result of dipolar interactions between transient *induced* dipole moments within atoms. Electrons in any given atom may at any point in time be non-uniformly distributed about the nucleus (although *on average* they *are* symmetrically displaced). This non-uniform distribution results in a momentary dipole. The resulting dipole can then induce a dipole in a neighboring atom, of opposite direction. This leads to an attractive force between the atoms. However, at shorter distances e^-e^- repulsion becomes greater than this attractive force, so that the atoms repel.

How important are van der Waals interactions to protein structure?

A single van der Waals interaction is only a few hundred calories, however, there are many such contacts in a single protein. So you can imagine that the energies could add up significantly. However, the folded and unfolded forms of the protein *both* have a large number of van der Waals contacts (the latter has many with water), so that the *net* stabilization of protein structure imparted by van der Waals interactions may be small.

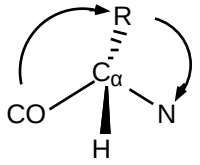
Bond stretching

Energies are too big to worry about. In other words, the energy cost to significantly lengthening (or shortening) a covalent bond in the final (average) protein structure is so high that such considerations can be ignored. Covalent bonds are considered to be constant in length and set at classic values for the bond involved.

Torsional constraints - Rotations around rotatable bonds

Aside - Stereochemistry at the C_α position

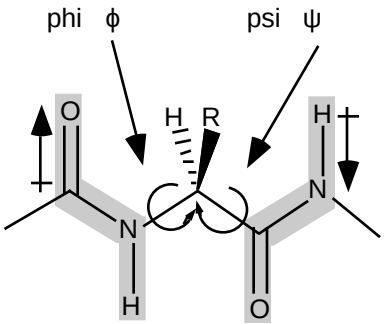
The stereochemistry at the C_α position can be easily remembered by use of the little corny pneumonic "CORN." With the (little) hydrogen pointing towards you, proceeding in a clockwise direction, the other three substituents should spell out "CORN" as shown at right (another contribution from Jane Richardson).

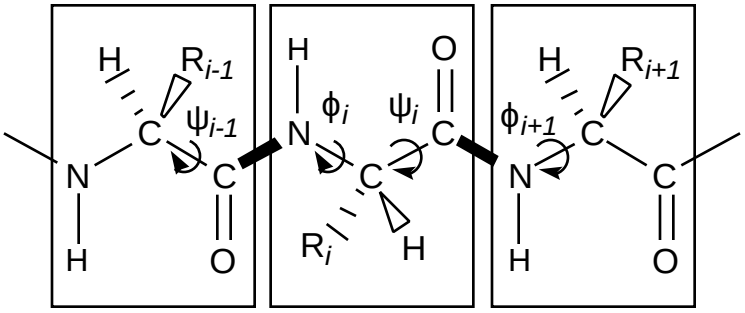


Rotation around some bonds is also too high in energy cost. For example, the peptide bond CO-NH has partial double bond character. For our purposes, then it can be assumed that rotation of the peptide bond from the angle at which the π system is planar costs too much energy to occur with any frequency.

However, rotation around the other two bonds in the polypeptide chain does occur at low energy. Consequently, variation in these angles will contribute to differences in protein structure. (see C&S pp 254-257 for a detailed explanation of the sign conventions).

ϕ ranges from -180 to 180° with 90° corresponding to a *cis* configuration. An all *trans* backbone (if it adopted the simple planar structure above) would correspond to ϕ and ψ = 180°.





For a protein α-helix, ϕ = -57° and ψ = -47° for every amino acid unit in the helix.

Ramachandran plots show that only certain ranges (and combinations of ranges) are allowed for these angles. This is a result of steric considerations of the functional groups. An important distinction is made for the amino acid glycine (Gly): Due to the very small size of its functional group (hydrogen), a much wider variety of angles is energetically reasonable. (see C&S p. 259 for illustration). This has important implications for protein folding.

Sterics effects possible phi / psi angles

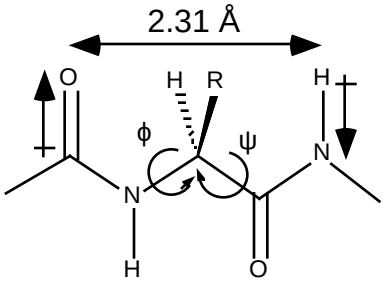
Of course, the major restriction on phi and psi angles arises due to the bulk of the groups attached to a given set of peptide bonds. Only certain ranges of angles allow low energy steric interactions.

Staggered vs. eclipsed

As we have all seen in organic chemistry classes, bonds prefer to rotate such that their attached bonds are "eclipsed." This is most simple explained in terms of sterics... Suffice it to say that this effects preferred values of phi and psi.

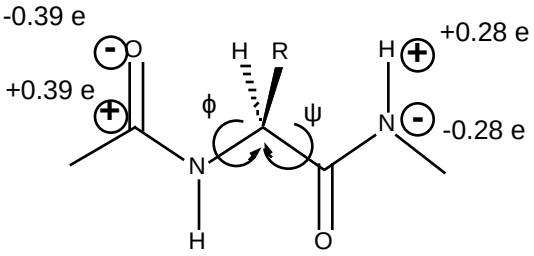
The dipolar interaction significantly effects Ramachandran values for phi and psi

The picture at right shows that the dipole moments associated with the peptide bond place energetic restrictions on the angles which can be adopted by phi and psi. Adjacent peptide bonds would like to interact favorably.

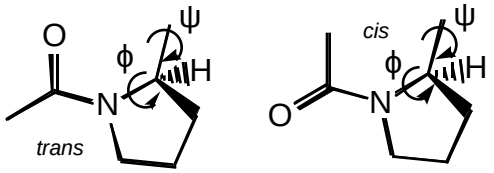


Effects of *unusual* amino acids on phi and psi

Gly has a simple hydrogen as its "functional group" (R above). Consequently the steric restrictions are much less for Gly and it is allowed a much wider range of phi/psi angles (see C&S).



Pro is also unusual in that the normal C_α-N bond is tied up in a 5-membered ring. Consequently the phi angle is rigidly fixed near -60°. The psi angle is not rigidly fixed, but is significantly more restricted than in any other amino acid. The angles for psi which result in energy minima are -55° (compact chain) and 145° (extended chain).



In addition, the psi angle at the amino acid *i-1* is more restricted and constrained to different values than in other contexts (the NH "upstream" of it is now N-R). So most amino acids are very sensitive to the presence of Pro at *i+1* (the exception is Gly, which already has a wide range of angles available to it and has little steric clash to present to the N-R group).

f

Covalent Interactions

As for bond stretching and amide bond rotation (but much more strongly), the disruption of a covalent bond requires a very high energy cost. Thus, covalent bonds are not considered as variable.

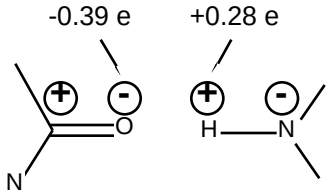
"Non-bonding" Interactions

A variety of non-bonding interactions contribute to protein stability, and in fact, it is these interactions, together with torsion angle changes which drive the formation of secondary and tertiary structure.

London dispersion energy -

Hydrogen bonding

One way of viewing hydrogen bonding is simple electrostatics. We could view it as a charge-charge attraction as shown at right. This is probably overly simplistic and does not include orbital overlap, which results in an angular dependence to the interaction.



Water - the unusual solvent

Compound	Melting point (K)	Boiling point (K)
H_2O	273	373
H_2S	190	211
$CH_3(CO)OH$	290	391
$CH_3(CO)CH_3$	178	330
CH_3CH_2OH	156	351
$CH_3CH_2CH_3$	83	231
CH_3OCH_3	135	249
CH_3NH_2	181	267
CH_3CH_3	101	185

Water structure - hydrophobic interactions

C&S Part I (pp 279-89) discuss hydrophobic interactions and water structure.

It's not as simple as they imply (see Dill below), but the concepts are interesting.

Water forms "cage-like lattices" around hydrophobic molecules

Water is indeed unique in its properties. Even in its liquid state, it adopts transient local "crystalline" structures (*microscopic icebergs*). ***Cage like lattices*** can form which can sequester small hydrophobic molecules. This results in enthalpic stabilization. But formation of these cage-like polyhedrons severely restricts the entropy of the water molecules - ie. entropically destabilized..

Hence there is a large entropic cost to the solvation of nonpolar molecules -- the hydrophobic effect is typically viewed as entropically driven.

Kauzman measured ΔH and ΔS for the transfer of a number of different molecules from nonpolar solvents to water (p. 285). ΔH is negative in all cases (the nonpolar molecules prefer water because the ice like structures create hydrogen bonds), but ΔS is negative as well (the nonpolar molecules decrease the entropy of the system in going to water and so prefer the non polar solvent). At room temperature, ΔG strongly favors transfer to the non polar solvent (entropically driven).

Denaturation by urea and guanidinium hydrochloride

Various hydrophobic or nonpolar side chains prefer 8 M urea to water. (that is $\Delta G_{tr\ water \rightarrow gua}$ is negative (similar arguments).

Ionic interactions

They occur in proteins. But are thought to be a minor contributor to stability (see below).

Like charges - repulsive - enthalpy unfavorable

Opposite charges - attractive - enthalpy favorable

A charge in aqueous solution requires substantial solvation. This decreases the entropy of the water. An ion pair requires much less.

Disulfide bonds

Significant entropic destabilization of the unfolded structure.

Recent studies suggest that disulfide bonds don't direct protein folding, but stabilize structures which already form. They stabilize the structure not so much by lowering the energy of the folded state, but by entropically raising the energy of the unfolded state.

Dominant Forces in Protein Folding? - Ken Dill Review (*Biochemistry 29*, 7133, 1991)

Electrostatic interactions

Long-range - if the net charge of a protein is non zero (ie the protein is on either side of its isoelectric point), then there will be a net repulsive interaction between unbalanced like charges. This forms the basis for the general trend that a protein most stable at its isoelectric point.

Specific charge interactions - attractive ion pairing (salt bridges, ionic bonds). Not too sensitive to bulk dielectric, since the interaction is short range - little intervening medium. Can effect unfolded state as well as folded state.

Most salt bridge interactions occur at the surface of the protein. The ion pair is not completely self-neutralizing and so requires solvation (cost to bury an ion pair is on the order of 7 kcal/mole). Rarely found in the interior of proteins. Any solvent effects which stabilize/destabilize ion pairs at the surface of the protein would have similar stabilizing/destabilizing effects on the ions in the unfolded state (little net effect). In fact recent studies suggest that hydration is better in the unfolded state (ion is more accessible).

Often associated with a change in partial molal volume. Isolated charges require extensive hydration. The water packs tightly around the charge - electrostriction.

Clearly ***not*** the dominant force in protein folding - protein stability is not a steep function around the pI, nor is stability strongly dependent on salt (dielectric of the medium).

Evolutionary studies show that surface charges are not highly conserved. Mutagenesis studies have confirmed that surface charges can usually be mutated with little perturbation of stability.

Too few to be a major net structural force (1-3 kcal/mole/ion pair, ≈5 ion pairs/150 aa's).

Hydrogen bonding and van der Waals interactions

H-bonding: discussed previously. View it either as 1) a local charge-charge attraction (with partial charges) or 2) as a bonding interaction involving orbital overlap.

van der Waals: Like H-bonding, it is short range and will have similar effects on energetics. Hard to model and understand the way we do H-bonding.

Helix-coil transitions: Balance between entropy and enthalpy. At low temperatures enthalpy dominates and the helix is favored. At high temperatures, entropy dominates and the random coil is favored. A sharp transition occurs. In general increased net charge of the protein should disfavor helix formation and favor the more expanded random coil. Solvents which H-bond well compete for the intrahelical H-bonding and so destabilize the helix.

Similar arguments for β-sheets.

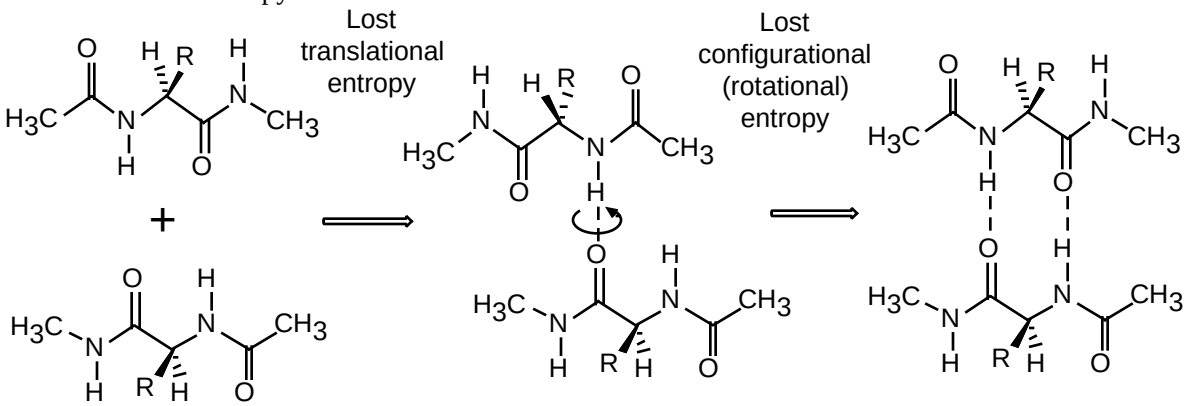
Kauzmann argued early on (and Dill agrees) that although H-bonding interactions are important to details of structure, they cannot drive protein folding.

The reason is that the H-bond interactions with water in the unfolded state are quite strong. The ***net*** change is very small and could go either way.

Cooperativity in formation of protein structures

The first H-bond in a unit of secondary structure typically costs energy due to loss of configurational entropy. But once that price is paid, subsequent H-bonds forms without the energetic expense. For this reason, helices become more stable and transitions sharpen with increasing length.

N-methyl-acetamide ($CH_3(CO)(NH)CH_3$) is a popular model to study effects on hydrogen bonding interactions (mimics the peptide backbone). At 25° C, dimerization is *disf*avored. Loss of translational entropy?



Additionally, consider entropic effects in the formation of multiple H-bonds. In the pairing of a dipeptide, two bonds can form in a single dimer. When only one forms, the two molecules still maintain a rotational degree of freedom about that bond. When the second bond forms, that rotation is completely lost - entropy decreases. Difficult to separate the effects.

Dill's conclusion: H-bonding alone will ***not*** drive protein folding, but if other processes favor folding, then H-bonds will be favorable within the structure.

Statistics from known protein structures:

- C=O groups:
  - 89% are H-bonded
    - 43% to water
    - 11% to side chains
    - 46% to main chain
- NH groups:
  - 88% are H-bonded
    - 21% to water
    - 11% to side chains
    - 68% to main chain

If H-bonding were dominant, then

- Solvents which are good H-bonders should destabilize protein structure, those that are bad H-bonders should have little effect. Observation: 1% SDS can denature proteins, despite its low concentration and the fact that it does *not* destabilize helices.
- Tetraalkylammonium salts increasing denature proteins as the length of the alkyl group increases (shouldn't effect H-bonding).
- Alcohols are more hydrophobic than water and they stabilize helices, yet they denature proteins.

Mutagenesis: H-bonds effect stability, but the magnitude and direction of the effect varies significantly.

"Local" interactions - "Intrinsic" properties (note his definition of "intrinsic")

- Must know the differential effect on native vs. unfolded state.
- Define local interactions as those occurring between an amino acid and another amino acid 3-4 distant in the primary sequence
- At 20° C, the propensity for a sequence to form a helix is generally quite small.
- Additional stability occurs if helix formation leads to burial of a nonpolar surface.
- Helices are further stabilized by charge compensation at the ends.
- Helices can be stabilized by salt bridges and other side chain interactions.
- Bottom line: context can be at least as important as "intrinsic" properties. Early on, people took sequences which were known to form helices in proteins and were somewhat surprised to find that they did not form helices when isolated in solution. Should be no surprise now.
- Helices in globular proteins are on average short (6-12 residues) - much shorter than should be stable. Conversely, the longer a helix, the more stable it should be, yet we don't see lots of long helices in solution (think about it - why?).
- Context "rules" for formation of helices in proteins (note that "rules" is in quotes...)
- Charges are distributed to stabilize the helix dipole moment (or the end carboxyl oxygen and amino proton).
- Ends of helices are often at the surface (same reason as above)
- Helices can pack in anti-aligned pairs to stabilize dipole moments (questionable importance)
- Other problems with "intrinsic" properties
- Cannot predict sheet structures, which are inherently non-local.
- Intrinsic properties have shown a 64% average success rate in predicting structure
- He goes through an analysis which says that this means local (intrinsic) factors contribute 15-30% of the total information required to predict a structure.

Hydrophobic effect - nonpolar solvation

- Nonpolar solvents denature proteins by lowering the energy of the unfolded state
- Evidence:
  1) Temp dependence of the free energy of folding follows that of transfer of nonpolar model compounds from water to nonpolar media
  2) Crystal structures confirm sequestering of nonpolar groups
  3) Dependence of stability on the nature of the salt species in solution follows the Hofmeister series, suggesting hydrophobic interactions
  4) Mutagenesis and other studies suggest that stability is proportional to the oil-water partitioning of the amino acid
  5) Hydrophobicity of core amino acids is evolutionarily conserved
  6) Computer simulations of incorrectly folded proteins shows that poor interior/exterior distribution of hydrophobic residues is a major factor in instability.

Intro to hydrophobic interactions

Note first that mixing of simple solutions is entropically driven. Molecules want to "spread out" and obtain the highest possible translational entropy (dispersion). However repulsion between molecules counters this driving force somewhat. When two components A and B are relatively insoluble in each other (strong repulsive forces between them), an enthalpic term opposes the entropic term. With increasing temperature, the entropic term dominates and the solute is eventually allowed to mix. Or rather, solubility goes up.

However, *entropic* terms can also oppose mixing. In water, nonpolar molecules are harbored in water "shells," the polyhedron we discussed before. The dissolution of a nonpolar molecule in the water, leads to extensive ordering of this solvation shell, and hence to a decrease in entropy of the system.

Narrow definition of hydrophobic interactions: Dill describes the hydrophobic interaction very narrowly as the transfer of a nonpolar solute to an aqueous solution only when the mixing is "***opposed by an excess entropy***."

"Excess entropy" is the entropy associated with transfer of a solute which is not derived from the simple (statistical) entropy of mixing.

$$\Delta S_{excess} = \Delta S_{total} - \Delta S_{ideal\_mixing}$$

Another property of the solvation of nonpolar solutes in water is that the transfer to water is characterized by an enthalpy *with a strong temperature dependence* (in other words, the heat capacity for transfer is large and positive).

Remember that $\Delta H = C_p \Delta T$

Now we have (assuming heat capacity independent of temperature::

$$d\Delta H = \Delta C_p dT$$

$$\int_{T_h}^{T} d\Delta H = \Delta C_p \int_{T_h}^{T} dT$$

$$\Delta H_T - \Delta H_{T_h} = \Delta C_p (T - T_h)$$

$$\Delta H_T = \Delta H_{T_h} + \Delta C_p (T - T_h)$$

$$d\Delta S = \frac{\Delta C_p dT}{T}$$

$$\int_{T_s}^{T} d\Delta S = \Delta C_p \int_{T_s}^{T} \frac{dT}{T}$$

$$\Delta S_T - \Delta S_{T_s} = \Delta C_p \ln\left(\frac{T}{T_s}\right)$$

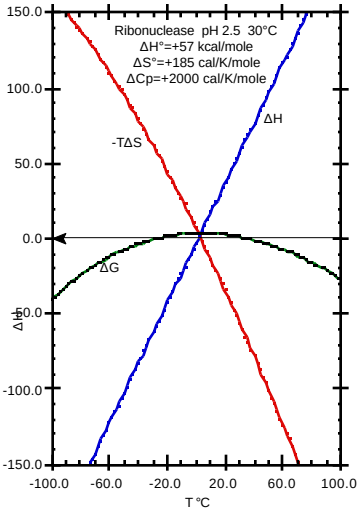
$$\Delta S_T = \Delta S_{T_s} + \Delta C_p \ln\left(\frac{T}{T_s}\right)$$

Therefore, assuming that the heat capacity is independent of temperature:

$$\Delta G_T = \Delta H_T - T\Delta S_T = \Delta H_{T_h} + \Delta C_p (T - T_h) - T\Delta S_{T_s} - T\Delta C_p \ln\left(\frac{T}{T_s}\right)$$

$$= \Delta H_{T_h} - T\Delta S_{T_s} + \Delta C_p \left(T - T_h - T \ln\left(\frac{T}{T_s}\right)\right)$$

This means that ΔG does not necessarily steadily decrease with temperature as would be expected for ΔG = ΔH - TΔS if ΔH and ΔS were temperature independent. The diagram at right shows that ΔG (for the *unfolding* of the protein) may reach a maximum at a particular temperature. Indeed, a similar plot can be drawn for the transfer of benzene to water, and benzene is least soluble in water near room temperature.



If protein folding were driven by this process, then we might expect proteins to exhibit a temperature at which the hydrophobic effect is maximal and the protein is therefore maximally stable (ie the protein would tend to unfold more readily at higher temperatures *and* at lower temperatures). This may be somewhat counterintuitive, but once you accept hydrophobicity as a major determinant of protein stability and the fact that the hydrophobic effect *weakens* at temperatures below its maximum, then you can see why some proteins are unstable at low temperatures - "cold denaturation." Of course for many proteins, the low end of stability may be below the freezing point of water and so cold denaturation is never observed.

This also means that the hydrophobic effect is entropy driven at lower temperatures, but enthalpy driven at higher temperatures! Why? Going back to the iceberg model for nonpolar solute solvation, at low temperatures the "cages" are well-formed and water molecules get to form lots of hydrogen bonds (ΔH happy), but there are few conformations available that optimally satisfy ΔH, so entropy is low (ΔS unhappy). At higher temperatures the opposite becomes true -- the clathrate structures break down (or rather they flex, "bending" the H-bonds), increasing entropy (ΔS happy), but at the cost of lost (or weaker) H-bonds (ΔH unhappy).

Bottom line: hydrophobic effects are characterized by a large heat capacity. Protein unfolding is typically characterized by a large heat capacity. Therefore protein unfolding must be accompanied (and by dominated by) the solvation (exposure) of nonpolar surfaces.

Residual enthalpy and entropy of unfolding

There is an additional positive entropy and enthalpy of unfolding, not accounted for by predictions from nonpolar solvation. From where do these arise? Residual enthalpy seems to increase with polar content - one proposal: folded protein has more H-bonding contacts. As for entropy, unfolding should result in increased entropy for the protein (bond angles - configurational entropy).

Site-directed mutagenesis studies - single amino acid substitutions

Various studies have now been done which involve the substitution of an interior amino acid in a protein by various other amino acids. If the system were simple, we could assume that the interior is represented by ethanol or a similar solvent, and that unfolding of the enzyme is a simple transfer from that solvent to water. To the extent that this is true, a plot of ΔΔG for each mutant as a function of ΔG_{tr} for that amino acid should yield a slope of 1. The non-ideality of this slope could arise from a number of factors:

1) "deformability" of the cavity in which the amino acid sits. How well does the protein adjust its local configuration to accommodate the substitution
2) specific interactions of each amino acid with the cavity, including enthalpy and entropy
3) how well does the denatured form of the protein mimic pure aqueous medium?
4) how exposed to water is the amino acid in the folded form (we assumed not at all)?

If the rest of the protein stabilizes a "cavity" into which the amino acid can fit, then the transfer of the side chain into that cavity will be less energetically costly than transferring the same side chain into the model solvent -- in the latter case, there is an energetic cost to create the cavity. Conversely, if the cavity is rigid and one tries to insert a larger amino acid by substitution, then the energetic cost would be higher than expected (slope > 1). These kinds of analyses have been done on a number of proteins, with slopes ranging from 1 to 4.

Summary: Definition of Hydrophobicity

Transfer of a nonpolar solute from non aqueous media to aqueous media when:

1) transfer is energetically strongly disfavored

and

2) transfer is associated with a large increase in heat capacity

Opposing Forces

If hydrophobicity were the only force involved, proteins would be much more stable than they are. What else is there?

Our discussion of hydrophobicity dealt with entropic effects from ordering water, but we ignored the large decrease in the entropy of the polypeptide associated with folding. We return to the entropy associated with rotation about the phi and psi bonds, and about the bonds in the amino acid side chains. He calls this “configurational” entropy. He also refers to this as “local” entropy.

Another aspect of the entropy loss in folding is what he calls “nonlocal” entropy. This has to do with the number of possible ways a polymer chain can fold onto itself. The hydrophobic driving force wants to minimize the exposure of nonpolar groups - there is only one (or maybe a few) configuration which minimizes that exposure. Consequently, on protein folding the chain folding is reduced from a very large number of possibilities down to 1 or a few.

We come back to things like disulfide linkages. These covalent linkages reduce the nonlocal entropy of the unfolded state -- there are now many fewer ways of folding the polymer chain in solution. Similarly, he argues that carbohydrates added to proteins can stabilize them by restricting the number of possible states in the unfolded form of the protein.

Internal architecture of proteins

To some extent, the internal architecture of proteins is dictated by this need to pack in the one form which will optimize burial of hydrophobic groups. But of course, we can also now see an important role for the H-bonding which is so readily observable. Which is dominant? Hard to say. Both are important. The upshot of this entire article is that although H-bonds are easy to see and to talk about, when all energies are added up, the hydrophobic effect is energetically the much larger force -- not the only force, but the more dominant one...

Analyses of Protein Folding

Two-State Model for Protein Folding

The basic model: N <-> D

Most protein unfolding studies assume a two-state model for denaturation. The protein can exist in only two discrete states: native (N) and denatured (D).

Multi-State Model for Protein Folding

Effect of multiple states on observables - what if unfolding is NOT two-state?

Assume the following scheme for protein unfolding:

$N \leftrightarrows I_1 \leftrightarrows I_2 \leftrightarrows \ldots \leftrightarrows I_n \leftrightarrows D$

where N refers to native and D to denatured.

Given a microscopic observable for each state y_i then the overall macroscopic observable y is given by:

$$y = f_N y_N + f_D y_D + \sum_{i=1}^{n} f_i y_i$$

where f_i is the mole fraction of proteins in state *i*. Then $f_N + f_D + \sum_{i=1}^{n} f_i = 1$

The observable can then be expressed as:

$$y = \left(1 - f_D - \sum_{i=1}^{n} f_i\right) y_N + f_D y_D + \sum_{i=1}^{n} f_i y_i$$

$$y = y_N - f_D y_N + f_D y_D + \sum_{i=1}^{n} \left(f_i y_i - f_i y_N\right)$$

If we measure our observable as before, assuming a two-state unfolding process, we will calculate an *apparent* fractional unfolding:

$$f_{app} = \frac{y - y_N}{y_D - y_N} = \frac{y_N - f_D y_N + f_D y_D + \sum_{i=1}^{n} \left(f_i y_i - f_i y_N\right) - y_N}{y_D - y_N}$$

$$f_{app} = \frac{f_D\left(y_D - y_N\right) + \sum_{i=1}^{n} \left(f_i y_i - f_i y_N\right)}{y_D - y_N}$$

$$f_{app} = f_D + \sum_{i=1}^{n} f_i \frac{y_i - y_N}{y_D - y_N}$$

$$f_{app} = f_D + \sum_{i=1}^{n} f_i d_i \quad \text{where} \quad d_i = \frac{y_i - y_N}{y_D - y_N}$$

from before, the apparent equilibrium constant for unfolding is: $K_{app} = \left(\frac{D}{N}\right)_{app} = \frac{f_{app}}{1 - f_{app}}$

We can also talk about (but maybe not measure) the true equilibrium constant: $K_D = \frac{D}{N} = \frac{f_D}{f_N}$

We can also talk about each microscopic equilibrium constant: $K_i = \frac{I_i}{N} = \frac{f_i}{f_N}$

$$\frac{f_{app}}{f_N} = \frac{f_D}{f_N} + \sum_{i=1}^{n} \frac{f_i}{f_N} d_i = K_D + \sum_{i=1}^{n} K_i d_i = K_D \left(1 + \sum_{i=1}^{n} \frac{K_i}{K_D} d_i\right)$$

$$\frac{1-f_{app}}{f_N} = \frac{\left(1 - f_D + \sum_{i=1}^{n} f_i d_i\right)}{f_N} = \frac{1-f_D}{f_N} - \sum_{i=1}^{n} \frac{f_i}{f_N} d_i$$

$$\frac{1-f_{app}}{f_N} = \frac{f_N + \sum_{i=1}^{n} f_i}{f_N} - \sum_{i=1}^{n} \frac{f_i}{f_N} d_i = 1 + \sum_{i=1}^{n} \left(\frac{f_i}{f_N} - \frac{f_i}{f_N} d_i\right) = 1 + \sum_{i=1}^{n} \left(\frac{f_i}{f_N}\left(1 - d_i\right)\right)$$

$$\frac{1-f_{app}}{f_N} = 1 + \sum_{i=1}^{n} \left[K_i\left(1 - d_i\right)\right]$$

$$K_{app} = \frac{f_{app}}{1-f_{app}} = \frac{\left(\frac{f_{app}}{f_N}\right)}{\left(\frac{1-f_{app}}{f_N}\right)} = \frac{K_D\left(1 + \sum_{i=1}^{n} \frac{K_i}{K_D} d_i\right)}{\left(1 + \sum_{i=1}^{n} \left[K_i\left(1 - d_i\right)\right]\right)} = K_D \frac{1 + \sum_{i=1}^{n} \frac{K_i}{K_D} d_i}{1 + \sum_{i=1}^{n} \left[K_i\left(1 - d_i\right)\right]}$$

Finally,

We have not yet said anything about how y_i depends on *i*. But let's look at two extremes, with one simple assumption.

The assumption: the observables for the intermediate states (y_i) lie between y_N and y_D.

Case 1: intermediate states have properties similar to the denatured state
then $y_i \approx y_D$ and $d_i \approx 1$

$$K_{app}\left(d_i \approx 1\right) = K_D \frac{1 + \sum_{i=1}^{n} \frac{K_i}{K_D}}{1 + 0} = K_D + \sum_{i=1}^{n} K_i$$

Case 2: intermediate states have properties similar to the native state
then $y_i \approx y_N$ and $d_i \approx 0$

$$K_{app}\left(d_i \approx 0\right) = K_D \frac{1}{1 + \sum_{i=1}^{n} K_i}$$

In any case, only when $K_i=0$ (no intermediates), then $K_{app}=K_D$.

In general, if intermediates exist, then K_{app} will depend on how d_i depends on the intermediate states. One way to test for intermediates is to measure K_{app} with different observables. If the two values of K_{app} are *not* the same, then K_{app} is not a true K_D - the process is not two state.

(Note from the extreme cases above, that the converse is not necessarily true).

Calorimetry

Similarly, we can worry about how the van't Hoff enthalpy that one has measured by the previous approaches is effected by the existence of intermediates.

Remember that

$$\Delta H = RT^2 \frac{\partial (\ln K)}{\partial T}$$

$$\Delta H_{app} = RT^2 \frac{\partial}{\partial T} \left[\ln K_D \frac{1 + \sum_{i=1}^{n} \frac{K_i}{K_D} d_i}{1 + \sum_{i=1}^{n} \left[K_i (1 - d_i) \right]} \right]$$

$$\Delta H_{app} = RT^2 \frac{\partial}{\partial T} \left[\ln K_D + \ln \left(1 + \sum_{i=1}^{n} \frac{K_i}{K_D} d_i \right) - \ln \left(1 + \sum_{i=1}^{n} \left[K_i (1 - d_i) \right] \right) \right]$$

$$\Delta H_{app} = RT^2 \frac{\partial \ln K_D}{\partial T} + RT^2 \frac{\partial}{\partial T} \ln \left(1 + \sum_{i=1}^{n} \frac{K_i}{K_D} d_i \right) - RT^2 \frac{\partial}{\partial T} \ln \left(1 + \sum_{i=1}^{n} \left[K_i (1 - d_i) \right] \right)$$

$$\Delta H_{app} = \Delta H_D + RT^2 \frac{1}{1 + \sum_{i=1}^{n} \frac{K_i}{K_D} d_i} \left(\frac{\partial}{\partial T} \sum_{i=1}^{n} \frac{K_i}{K_D} d_i \right) - RT^2 \frac{1}{1 + \sum_{i=1}^{n} \left[K_i (1 - d_i) \right]} \left(\frac{\partial}{\partial T} \sum_{i=1}^{n} \left[K_i (1 - d_i) \right] \right)$$

$$\Delta H_{app} = \Delta H_D + RT^2 \frac{1}{1 + \sum_{i=1}^{n} \frac{K_i}{K_D} d_i} \left(\sum_{i=1}^{n} \frac{\partial K_i}{\partial T} \frac{1}{K_D} d_i - \frac{\partial K_D}{\partial T} \frac{K_i}{K_D^2} d_i \right) - RT^2 \frac{1}{1 + \sum_{i=1}^{n} \left[K_i (1 - d_i) \right]} \left(\sum_{i=1}^{n} \frac{\partial K_i}{\partial T} (1 - d_i) \right)$$

Remember that: $\Delta H = RT^2 \frac{\partial (\ln K)}{\partial T} = RT^2 \frac{1}{K} \frac{\partial K}{\partial T}$ so that $\frac{\partial K}{\partial T} = \frac{K}{RT^2} \Delta H$

Then

$$\Delta H_{app} = \Delta H_D + \frac{1}{1 + \sum_{i=1}^{n} \frac{K_i}{K_D} d_i} \left(\sum_{i=1}^{n} \frac{\Delta H_i K_i}{K_D} d_i - \Delta H_D \frac{K_i}{K_D} d_i \right) - \frac{1}{1 + \sum_{i=1}^{n} \left[K_i (1 - d_i) \right]} \left(\sum_{i=1}^{n} \left[\Delta H_i K_i (1 - d_i) \right] \right)$$

$$\Delta H_{app} = \Delta H_D + \frac{\sum_{i=1}^{n} \left(\Delta H_i - \Delta H_D \right) \frac{K_i}{K_D} d_i}{1 + \sum_{i=1}^{n} \frac{K_i}{K_D} d_i} - \frac{\sum_{i=1}^{n} \left[\Delta H_i K_i (1 - d_i) \right]}{1 + \sum_{i=1}^{n} \left[K_i (1 - d_i) \right]}$$

$$\Delta H_{app} = \Delta H_D \left[1 - \frac{\sum_{i=1}^{n} \left(1 - \frac{\Delta H_i}{\Delta H_D} \right) \frac{K_i}{K_D} d_i}{1 + \sum_{i=1}^{n} \frac{K_i}{K_D} d_i} - \frac{\sum_{i=1}^{n} \frac{\Delta H_i}{\Delta H_D} \left[K_i (1 - d_i) \right]}{1 + \sum_{i=1}^{n} \left[K_i (1 - d_i) \right]} \right]$$

a

gain, as expected if K_i are all zero (two state process), then $\Delta H_{app} = \Delta H_D$.

Again, however, if all of the d_i are zero but K_i are not, we have:

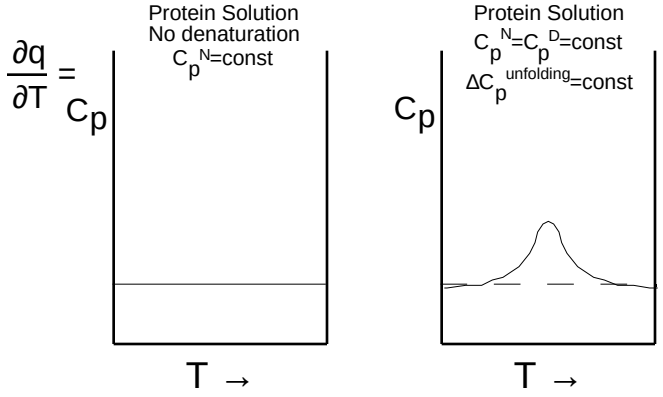
$$\Delta H_{app} = \Delta H_D \left[1 - \frac{\sum_{i=1}^{n} \frac{\Delta H_i}{\Delta H_D} K_i}{1 + \sum_{i=1}^{n} K_i} \right]$$

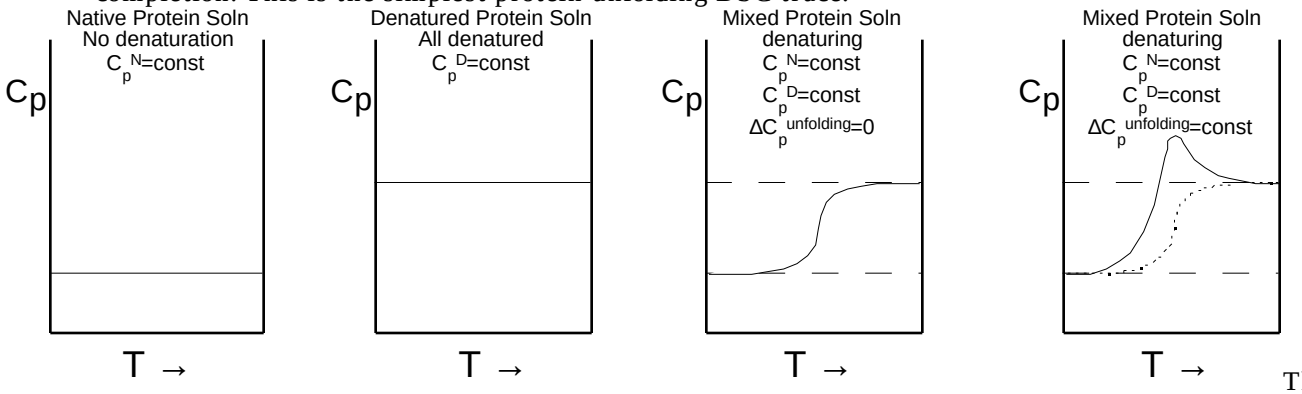
And, again $\Delta H_{app} \neq \Delta H_D$.

Differential Scanning Calorimetry

The method

The plots at right show a measurement of the heat capacity of a solution of protein. The plot to the left just shows the DSC scan for a solution of constant heat capacity, with no reaction proceeding. The plot to its right shows the scan for a solution with constant solution heat capacity, but for which a reaction with non-zero heat capacity is proceeding with increasing temperature. At the beginning of the reaction, the total heat capacity of the solution rises above that for the solution with no reaction, reaching a maximum at the midpoint of the reaction, and decreasing as the reaction goes to completion. This is the simplest protein unfolding DSC trace.





The traces above show first two different solutions in which no reaction is occurring. As we discussed previously, the solution with denatured protein typically has a higher heat capacity than that with native protein. If the unfolding reaction had zero ΔH associated with it, we could simply use the change in heat capacity of the solution as a function of the percent unfolded to monitor unfolding in the same way that we used CD spectra or other techniques to monitor unfolding. An observable changes with protein unfolding. This is shown in the third panel above.

Finally, if the reaction has an enthalpy associated with it, we observe a combination of these two effects on heat capacity of the total solution illustrated at far right.

Derive an expression for total measured heat capacity as a function of the extent of the reaction (which is a function of temperature).

Assume heat capacity independent of temperature!!

Assume that the heat capacity of a mixture of native and denatured protein is simply the mole fraction weighted average of the two heat capacities.

Begin with the following parameters:

C_p^D = molar heat capacity of a solution of denatured protein

C_p^N = molar heat capacity of a solution of native protein

We also have given:

$\Delta H^o_{T_o}$ = molar enthalpy for unfolding of the protein at T = T_o

$\Delta S^o_{T_o}$ = molar entropy for unfolding of the protein at T = T_o

The total heat capacity for the solution is:

$\frac{\Delta q}{\Delta T}(measured)$

= (heat from heat capacity of denatured protein solution)

+(heat from heat capacity of native protein solution)

+(heat from that fraction of protein which denatured during ΔT)

$$\frac{\Delta q}{\Delta T}(measured) = \left(f_D C_p^D + f_N C_p^N + \Delta f \frac{\Delta H}{\Delta T}\right)[protein]$$

where we now remember that the enthalpy (ΔH) is temperature dependent.

First, let's determine the temperature dependence of ΔH, ΔS, ΔG, K_D, and f:

We know that:

$$\partial \Delta H = \Delta C_p \partial T \quad \partial \ln K = \frac{-\Delta H_{(T)}}{R} \partial\left(\frac{1}{T}\right)$$

integrating from one temperature to another:

$$\int_{T_1}^{T_2} \partial \Delta H = \int_{T_1}^{T_2} \Delta C_p \partial T \qquad \Delta H_{T_2} - \Delta H_{T_1} = \Delta C_p \left(T_2 - T_1\right) \qquad \Delta H_T = \Delta H_{T_o} + \Delta C_p \left(T - T_o\right)$$

Similarly,

$$\int_{T_1}^{T_2} \partial \Delta S = \int_{T_1}^{T_2} \Delta C_p \frac{\partial T}{T} \qquad \Delta S_{T_2} - \Delta S_{T_1} = \Delta C_p \ln \frac{T_2}{T_1} \qquad \Delta S_T = \Delta S_{T_o} + \Delta C_p \ln \frac{T}{T_o}$$

and

$$\Delta G_T = \Delta H_T - T\Delta S_T$$

$$\Delta G_T = \left[\Delta H_{T_o} + \Delta C_p \left(T - T_o\right)\right] - T\left(\Delta S_{T_o} + \Delta C_p \ln \frac{T}{T_o}\right)$$

We can get the equilibrium constant, K from $K = e^{-\Delta G / RT}$

So, we have the extent of the reaction given by f (mole fraction protein unfolded), which can be derived as:

$$K = \frac{[unfolded\,]}{[\,folded\,]} = \frac{f}{1-f} \qquad \text{so that} \qquad f = \frac{K}{1+K}$$

Finally,

$$\frac{\Delta q}{\Delta T}(measured) = \left(f C_p^D + (1-f) C_p^N + \Delta f \frac{\Delta H}{\Delta T}\right)[protein]$$

$$= \left(f C_p^D + C_p^N - f C_p^N + \Delta f \frac{\Delta H}{\Delta T}\right)[protein]$$

$$= \left(f\left(C_p^D - C_p^N\right) + C_p^N + \Delta f \frac{\Delta H}{\Delta T}\right)[protein]$$

$$= \left(f \Delta C_p + C_p^N + \Delta f \frac{\Delta H}{\Delta T}\right)[protein]$$

DNA Melting / Strand Association

DNA duplex formation/melting is particularly amenable to simple thermodynamics.

$\Delta C_p = 0$ for this process. Hence not only are basic assumptions held up, but ΔH^o and ΔS^o are temperature independent.

Strand association appears to be dominated by "nucleation." It can usually be treated as a simple two-state process. Again, assumptions are valid.

Thermodynamics - Two non-*self*-complementary, complementary strands

$$S_1 + S_2 \leftrightarrows D \qquad K = \frac{D}{S_1 S_2}$$

Assume that when fully melted, $S_1 = S_2 = S_o$.

At any temperature, the fraction associated can be represented as α.

$$D = \alpha S_o \qquad S_1 = S_2 = (1-\alpha) S_o$$

$$K = \frac{\alpha S_o}{(1-\alpha)^2 S_o^2} = \frac{\alpha}{(1-\alpha)^2} \frac{1}{S_o}$$

Remembering $\Delta H_{vH} = RT^2 \frac{\partial \ln K}{\partial T}$

$$\Delta H_{vH} = RT^2 \frac{\partial}{\partial T}\left[\ln \alpha - 2\ln(1-\alpha) - \ln S_o\right]$$

$$\Delta H_{vH} = RT^2 \left[\frac{1}{\alpha} + \frac{2}{1-\alpha}\right]\frac{\partial \alpha}{\partial T} = RT^2 \left[\frac{1-\alpha+2\alpha}{\alpha(1-\alpha)}\right]\frac{\partial \alpha}{\partial T} = RT^2 \left[\frac{1+\alpha}{\alpha(1-\alpha)}\right]\frac{\partial \alpha}{\partial T}$$

At the T_m, α=1/2, such that

$$\Delta H_{vH} = RT_m^2 \left[\frac{3/2}{1/2 \cdot 1/2}\right]\left(\frac{\partial \alpha}{\partial T}\right)_{T_m} = 6\,RT_m^2 \left(\frac{\partial \alpha}{\partial T}\right)_{T_m} = -6R\left(\frac{\partial \alpha}{\partial\left(\frac{1}{T}\right)}\right)_{T_m}$$

Plot α vs. 1/T and plot according to the above equation. This yields ΔH_{vH}.

Thermodynamics - Two *self*-complementary strands

as above, but now S_o = total conc of ss DNA

$S_1 + S_2 = S_o \qquad S_1 = S_2 = \frac{1}{2}(1-\alpha)S_o \qquad D = \frac{1}{2}\alpha S_o$

$$K = \frac{\frac{1}{2}\alpha S_o}{\left(\frac{1}{2}\right)^2 (1-\alpha)^2 S_o^2} = \frac{\alpha}{(1-\alpha)^2}\frac{1}{2S_o}$$

as before:

$$\Delta H_{vH} = RT^2 \frac{\partial}{\partial T}\left[\ln\alpha - 2\ln(1-\alpha) - \ln S_o - \ln 2\right]$$

$$\Delta H_{vH} = RT^2\left[\frac{1}{\alpha} + \frac{2}{1-\alpha}\right]\frac{\partial\alpha}{\partial T} = RT^2\left[\frac{1-\alpha+2\alpha}{\alpha(1-\alpha)}\right]\frac{\partial\alpha}{\partial T} = RT^2\left[\frac{1+\alpha}{\alpha(1-\alpha)}\right]\frac{\partial\alpha}{\partial T}$$

Exactly as before.

Generalities

Given that ΔC_p=0, we can very simply extrapolate from a set of data

The temperature dependence of the equilibrium constant is given by: $\ln K = \frac{-\Delta H^\circ}{R}\left(\frac{1}{T} - \frac{1}{T_m}\right)$

And from this we can readily get to ΔG° and ΔS°.

Breslauer *et al.*

From Breslauer et al., Proc. Natl. Acad. Sci. U.S.A. 83, 3746-3750 (1986).

All data for DNA in 1 M NaCl, pH 7, 25°C

Dinucleotide Step		ΔH°	ΔS°	ΔG°
AA/TT	TT/AA	9.1	24.0	1.9
AT/TA	-	8.6	23.9	1.5
TA/AT	-	6.0	16.9	0.9
CA/GT	TG/AC	5.8	12.9	1.9
GT/CA	AC/TG	6.5	17.3	1.3
CT/GA	AG/TC	7.8	20.8	1.6
GA/CT	TC/AG	5.6	13.5	1.6
CG/GC	-	11.9	27.8	3.6
GC/CG	-	11.1	26.7	3.1
GG/CC	CC/GG	11.0	26.6	3.1

ΔH° and ΔG° in kcal/mole; ΔS° in cal/K/mole

For non-self-complementary, but complementary duplexes: $\Delta H_{total} = \approx 0 + \Sigma\,\Delta h_x$

For non-self-complementary, but complementary duplexes: ΔG_{total} = -6 kcal/mole* + $\Sigma\,\Delta g_x$

*The free energy of duplex nucleation is estimated at -6 kcal/mole for non-self-complementary duplexes and -5 kcal/mole for self-complementary duplexes. Empirically determined?

Example: Duplex: CACTATA

ΔH_{total} = 0 + 5.8 + 6.5 + 7.8 + 6.0 + 8.6 + 6.0 = 40.7 kcal / mole

ΔG_{total} = -6 + 1.9 + 1.3 + 1.6 + 0.9 + 1.5 + 0.9 = 2.1 kcal / mole

By this accounting, ΔS° should be (ΔH° - ΔG°)/(298 K) = 129.5 cal / K / mole

$\Sigma\,\Delta s_x = \Delta s_{init}$ + 12.9 + 17.3 + 20.8 + 16.9 + 23.9 + 16.9 = Δs_{init} + 35.1 cal / K / mole

Therefore Δs_{init} = 94.4 cal / K / mole

Also, this predicts: $T_m = \Delta H/\Delta S$ = 314 K (41°C)

Can we use this to predict a T_m?

For equal concentrations of **self-complementary** strands:

$S_1 + S_2 = S_o \quad S_1 = S_2 = \frac{1}{2}(1-\alpha)S_o \qquad D = \frac{1}{2}\alpha S_o \qquad K = \frac{\alpha}{(1-\alpha)^2}\frac{1}{2S_o}$

At the midpoint of the melting transition, α=1/2 $K_{T_m} = \frac{1/2}{(1-1/2)^2}\frac{1}{2S_o} = \frac{1}{S_o}$

$-RT_m \ln K_{T_m} = -RT_m \ln\left(\frac{1}{S_o}\right) = \Delta H - T_m \Delta S \qquad T_m = \frac{\Delta H}{\Delta S - R\ln\left(\frac{1}{S_o}\right)}$

For equal concentrations of **non-self-complementary, but complementary** strands:

$D = \alpha S_o \qquad S_1 = S_2 = (1-\alpha)S_o \qquad K = \frac{\alpha S_o}{(1-\alpha)^2 S_o^2} = \frac{\alpha}{(1-\alpha)^2}\frac{1}{S_o}$

At the midpoint of the melting transition, α=1/2 $K_{T_m} = \frac{1/2}{(1-1/2)^2}\frac{1}{S_o} = \frac{2}{S_o}$

$-RT_m \ln K_{T_m} = -RT_m \ln\left(\frac{2}{S_o}\right) = \Delta H - T_m \Delta S \qquad T_m = \frac{\Delta H}{\Delta S - R\ln\left(\frac{2}{S_o}\right)}$

$$T_m = \frac{\Delta H}{\Delta S - R\ln\left(\frac{2}{S_o}\right)} = \frac{40700 cal/mol}{(129.5 cal/K/mol) - (1.987 cal/K/mol)\ln\left(\frac{2}{S_o}\right)}$$

Assume $[S_o]$=20 nM, then $T_m = \frac{40700 cal/mol}{(129.5 cal/K/mol) - (1.987 cal/K/mol)\ln\left(\frac{2}{10^{-8}}\right)}$

However, remember our error analysis (**This needs to be corrected slightly** - see above):

$$T_m = \frac{\Delta H}{\Delta S} = \frac{T\Delta H}{\Delta H - \Delta G}$$

$$\Delta T_m^2 = \left|\frac{T}{\Delta H - \Delta G} - \frac{T\Delta H}{(\Delta H - \Delta G)^2}\right|^2 \Delta\Delta H^2 + \left|\frac{-T\Delta H}{(\Delta H - \Delta G)^2}\right|^2 \Delta\Delta G^2$$

$$\Delta T_m^2 = \left|\frac{-T\Delta G}{(\Delta H - \Delta G)^2}\right|^2 \Delta\Delta H^2 + \left|\frac{-T\Delta H}{(\Delta H - \Delta G)^2}\right|^2 \Delta\Delta G^2$$

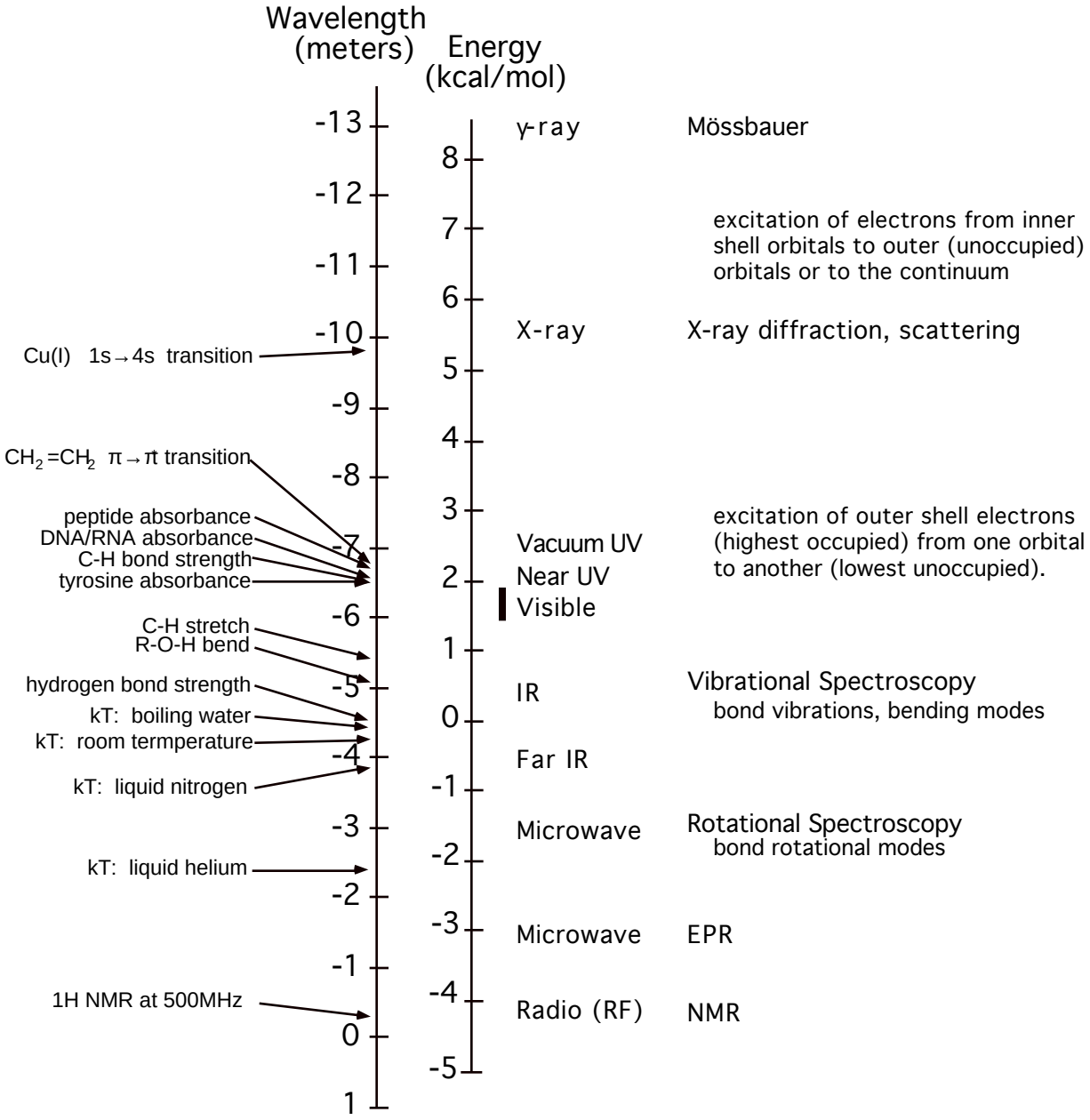
where ΔΔH and ΔΔG refer to the errors in our estimations of ΔH and ΔG, respectively. Assume that they are: ΔΔH = 2 kcal/mole, ΔΔG = 1 kcal/mole

Then

$$\Delta T_m = \left|\frac{-(298)2.1}{(38.6)^2}\right|2 + \left|\frac{-(298)40.7}{(38.6)^2}\right|1 = 0.84 + 8.1 = 9.0K$$

General Properties of Light and Matter (VH 8; CS 7)

Range of energy: spectroscopies and molecular processes



very high energies - electrons from inner to extreme outer shells or to continuum
x-rays: scattering from atomic centers
UV: excitation of electrons from one orbital to another
visible: excitation of electrons from one "big" orbital to another
IR: bond vibrations, bends
microwave: bond rotational modes, EPR
RF: nuclear magnetic resonance

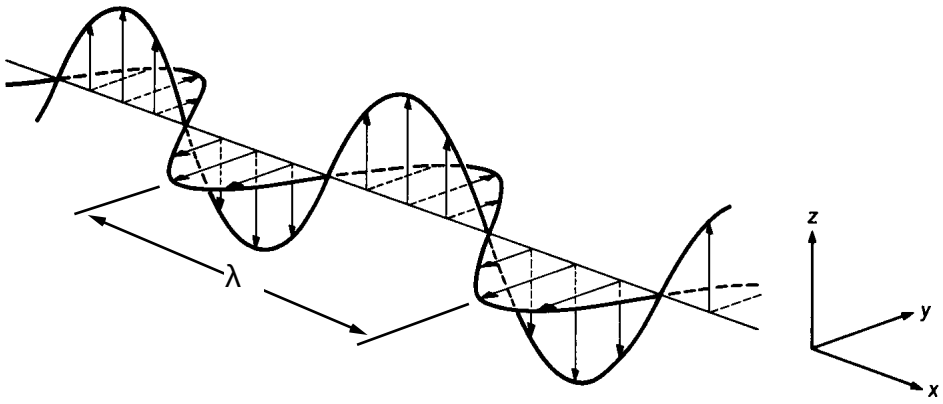
"Light" and energy

$$E = h\nu = hc/\lambda$$

$h = 6.63 \times 10^{-27}$ erg s $c = 3.0 \times 10^{10}$ cm s^{-1} $N_o = 6.02 \times 10^{23}$ $mole^{-1}$

$m_e = 9.11 \times 10^{-28}$ g; $k = 1.38 \times 10^{-16}$ erg K^{-1} $R = N_o k = 1.987$ cal mol^{-1} K^{-1}

erg = g cm^2 s^{-2} cal = 4.18×10^7 erg $/h = 2\pi h$ $\nu = 2\pi\omega$



Plane-polarized radiation

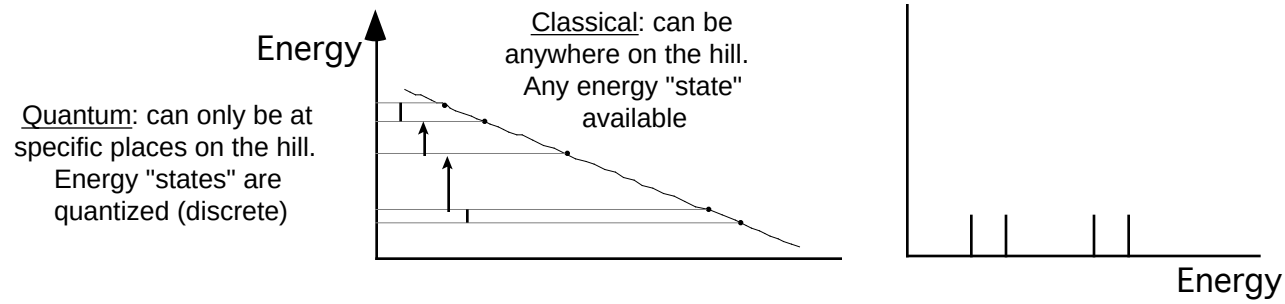
Electromagnetic radiation ("light") composed of electrical and magnetic components.

E and B components are perpendicular and vary in space with time. Light can be represented as a spatial "wave." Frequency of oscillation is ν, and wavelength λ.

picture here....

This is the classical representation and we will mostly stick with this kind of explanation, however, there are times when classical analogies fail, and only quantum mechanics will correctly predict nature.

Quantization of energy - only discrete states available



Quantum mechanics tells us that systems can only exist in discrete states.
There is no classical analogy here.
In classical mechanics, a **spring** of a specified length, width, and physical makeup can vibrate at any of an infinite number of frequencies. In quantum mechanics only discrete states are available, which are defined by the physical properties of the spring. Since states are discrete, energy gaps are discrete, and spectroscopic band widths *would* be very narrow, but heterogeneity in sample and environment broadens most bands significantly.

Each component in an ensemble has a discrete transition energy, but heterogeneity within the ensemble of microsystems results in a broad band associated with the macrosystem.
Later: Dynamics will also be seen to effect linewidths.
We will also see later that spin states of nuclei and electrons are also quantized ("up" and "down").

Brief summary of quantum mechanics

The state of a system (atom, molecule) is described by a <u>wave function</u>

ψ(x,y,z,spin,t) (ψ is, in general, a complex function - imaginary numbers...)

The probability of finding the system at a particular set of conditions of a state is given by the product of ψ and its complex conjugate ψ^*. $P = \psi\,\psi^*$

The probability of the system being "anywhere" in state ψ is determined by averaging over all conditions of that state: $P(t) = \int P\,dx\,dy\,dz\,d(spin) = \int \psi\,\psi^*\,d\tau$. Because QM tells us that systems exist discretely in a given state, then $\int \psi\,\psi^*\,d\tau = \langle\psi|\psi\rangle = 1$. ($d\tau$ means "over all space - dx dy dz)

Operators - The Result of a Measurement

An observable quantity (e.g. energy, dipole moment, location in space) is governed by a mathematical device known as an operator.

The Hamiltonian H is used to describe the energy of a state.

The result of a measurement on a state (e.g. measure the energy) can be calculated by taking the average value of the operator operating on that state: the expectation value.

$$\int_{-\infty}^{\infty}\int_{-\infty}^{\infty}\int_{-\infty}^{\infty} \psi\,H\,\psi\;dx\,dy\,dz = \int \psi\,H\,\psi\;d\tau = E \qquad \text{in other words,} \qquad \langle\psi|H|\psi\rangle = E$$

A transition between two states can be *induced* by a perturbation.

The effectiveness of this induction is governed by the extent to which the perturbation can deform the initial state to make it resemble the final state (*i.e.*, mix the states)

Assume a perturbation which can *mix* states, described as a potential **V**. The expectation that it will induce a system in state ψ_1 to effect a transition to state ψ_2 is given by

$P(\text{mixing}) = \langle\psi_2|\mathbf{V}|\psi_1\rangle$ remember that as $\mathbf{V}|\psi_1 \rightarrow \psi_2$, then this goes to $\langle\psi_2|\psi_2\rangle = 1$

Light can be a perturbation

The ability of light to induce transitions can be calculated by its ability to induce dipole moments that oscillate with the light.

The electric component of light can induce dipoles in electronic states.

In this case, the probability of an electric vector $\boldsymbol{\mu}$ inducing a transition is $\langle\psi_2|\boldsymbol{\mu}|\psi_1\rangle$

Perturbations have a directionality

The preferred directions for inducing dipole moments are determined by and fixed with respect to the geometry of the molecule.

ψ is a function of space (x,y,z), as is $\boldsymbol{\mu}$, therefore the ability of the electric dipole (vector) to induce transitions is dependent on their relative geometries.

Light can *induce* transitions between states (CS 7.1)

Energy match is required

Probability of light-induced transition is related to the matching of the light energy to the energy difference between levels

From quantum mechanics, the probability of a system initially in state *a* being found in state *b* (ie. a→b) is given by the following:

$$P_b = |C_b(t)|^2 = \frac{|\langle\psi_b|\boldsymbol{\mu}|\psi_a\rangle\cdot\mathbf{E}_o|^2}{\hbar^2}\;\frac{t^2\sin^2[((E_b-E_a)/\hbar-\omega)\,t/2]}{2[((E_b-E_a)/\hbar-\omega)\,t/2]^2}$$

$\boldsymbol{\mu}$ induces a dipole in state a, perturbing it to "resemble" state b. This term reflects this ability.

as this term → 0, $P_b \rightarrow 1$

where $\boldsymbol{\mu}$ is the electric dipole operator, $\mathbf{E_o}$ is the electric field (a vector), and E_b and E_a are the energies of systems in states ψ_b and ψ_a, respectively.

Note that since $\hbar\omega$ is the energy of the light, transitions from *a* to *b* will be most likely when the denominator $((E_b-E_a)/\hbar-\omega)$ is small, that is when

$h\nu = \hbar\omega = E_b - E_a$ energy of light matches energy difference between states

The amount of *net* light absorption is dependent on the number of molecules in each state

It can be shown that for radiation centered at frequency ν, the rate at which molecules are transformed from state *a* to state *b* is given by

$$\frac{dP_b}{dt} = \frac{d}{dt}\int d\nu\, |C_b(t)|^2 = \frac{1}{2\hbar^2}\, |\langle\psi_b|\underline{\boldsymbol{\mu}}|\psi_a\rangle \cdot \mathbf{E}_o|^2$$

for polarized light; a specific orientation of the molecule, and single frequency ν.

More generally, integrating over all orientations, it can be shown that

$$|\langle\psi_b|\underline{\boldsymbol{\mu}}|\psi_a\rangle \cdot \mathbf{E}_o|^2 = (1/3)\, |\langle\psi_b|\underline{\boldsymbol{\mu}}|\psi_a\rangle|^2\, |\mathbf{E}_o|^2$$

then $$\frac{dP_b}{dt} = \frac{1}{6\hbar^2}\, |\langle\psi_b|\underline{\boldsymbol{\mu}}|\psi_a\rangle|^2\, |\mathbf{E}_o|^2$$

From classical E & M:

$$I(\nu) = \frac{|\mathbf{E}_o|^2}{4\pi}$$ Intensity: energy density incident on the sample at frequency ν

We can then define

$$B_{ab} = \frac{2}{3}\frac{\pi}{\hbar^2}\, |\langle\psi_b|\underline{\boldsymbol{\mu}}|\psi_a\rangle|^2$$ transition rate ($a \rightarrow b$) per unit energy density

So that $\frac{dP_b}{dt} = B_{ab}\, I(\nu)$ the two components are separable

Note: V = k [C] analogy to a first order kinetic "reaction"

This result is for any system initially in state *a*. It defines the probability of finding that system in state *b* at some time t (as a result of interaction of the system with the electric field component of the light. It contains two components: 1) B_{ab}, the transition rate (analogous to a chemical rate constant) and 2) $I(\nu)$, the energy density, or intensity, of the light - roughly the density of photons hitting the sample (analogous to "concentration of photons"). Note that at this point we have said nothing about which state is higher in energy.

A similar expression can be written for the transition from state *b* to state *a*. So that light is both emitted and absorbed. The *net* change then depends on the population of each level, such that the *net* absorption of light can be written as:

$$-\frac{dI(\nu)}{dt} = h\nu\,(N_a B_{ab} - N_b B_{ba})\, I(\nu)$$ The rate at which energy is removed from the light

where $h\nu = E_b - E_a$ (the energy of the transition between states)
For simple cases of interest to us, the Einstein coefficients $B_{ab} = B_{ba}$, so that

$$-\frac{dI(\nu)}{dt} = h\nu\,(N_a - N_b)\;B_{ab}\;I(\nu) \qquad \leftarrow \textbf{N.B.}$$

Thus, through the factors N_a and N_b, light absorption depends not only on the concentration of the species, but also on the <u>difference in population</u> between the levels. A *very* important result of this is that no matter how much light you shine on the system, the most you can ever do is equally populate each of the levels. You can not (without getting fancy) "pump" all of the systems from state *a* to state *b*. We will see this later.

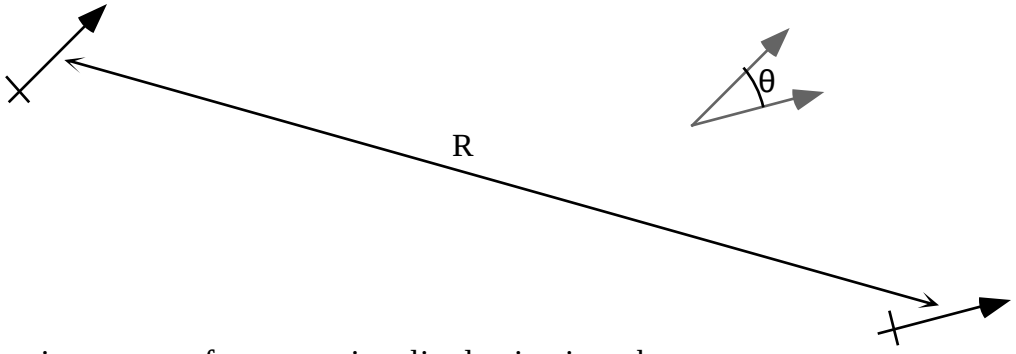
Light induces dipoles in molecules

$B_{ab} \propto \langle\psi_b|\boldsymbol{\mu}|\psi_a\rangle$ = the dipole moment induced by the light = <u>transition dipole moment</u>
Classically, electromagnetic radiation (light) possesses an electric field component, $\mathbf{E}_o$ above. Since light is an oscillating wave, the electric field also oscillates. The oscillating field can then interact with an existing dipole or induce an oscillating dipole in the molecule, $\boldsymbol{\mu}$ above.
The integral $\langle\psi_b|\boldsymbol{\mu}|\psi_a\rangle$ (<u>transition dipole moment</u>, it is a vector) describes the ability of light to distort a molecule in state *a* so as to produce a system which resembles state *b*.
Oscillations have a time component, and so have *phase*. This will be important when two dipoles are simultaneously excited. Relative phases important.

Transitions induced in *both directions* equally.

Purely a result of quantum mechanics
Net absorption *usually* observed, due to population of states (see below).

Dipole-dipole interaction (CS p. 263-265)



The interaction energy for two point dipoles is given by:

$$E_d = \varepsilon^{-1}\left[\frac{\boldsymbol{\mu}_A \cdot \boldsymbol{\mu}_B}{r^3} - \frac{3\,(\boldsymbol{\mu}_A \cdot \mathbf{r})\,(\boldsymbol{\mu}_A \cdot \mathbf{r})}{r^5}\right]$$

In this *point dipole* approximation, the charge separation (distance) within each dipole is assumed to be much smaller than the distance separating the two dipoles. This is not necessarily true in chemical systems, but a more rigorous calculation is generally prohibitive. A further approximation is generally made in dropping the second term in the equation. This assumption is generally not too bad.
Note that the <u>dielectric</u> constant, ε, is involved. Pictorially, this means that if the intervening medium has its own dipoles between the two point dipoles, their effect will lessen the interaction between the two dipoles.
Note also that there is an <u>angular dependence</u>, such that dipoles oriented 90° with respect to one another have no interaction energy.

Finally, the large distance dependence ($\frac{1}{r^3}$) in this equation shows that only near neighbor atoms in a molecule interact substantially.

Boltzmann distribution (VH 2; CS 8.2)

Boltzmann equation specifies thermal distribution:

$$n_b = n_a\, e^{\frac{-(E_b - E_a)}{kT}} \qquad \frac{n_b}{n_a} = e^{\frac{-\Delta E}{kT}}$$

Thermal energy allows for transitions between states, but this is, of course, limited by the energy separation of the states relative to the thermal energy (*ie.* the temperature). The temperature equivalent of photons are called phonons. Thermal energy (the phonon bath) generally spans a wide range of energies, and so heat can usually bring a system to thermal equilibration.

Implications:

For states separated by substantial energy gap, lower energy state will be exclusively populated, *i.e.* as $\Delta E \rightarrow \infty$, $n_b/n_a \rightarrow 0$.

For closely spaced states, populations can be *almost* evenly distributed, *i.e.* as $\Delta E \rightarrow 0$, $n_b/n_a \rightarrow 1$.

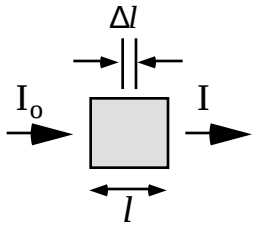
Temperature dependence: as $T \rightarrow 0$ (K), high energy states depopulated, $n_b/n_a \rightarrow 0$.

Optical Spectroscopies

Absorption Spectroscopy

Beer-Lambert Law (CS 7.2)

The fraction of light absorbed:



$$\frac{-\Delta I}{I} = C\,\varepsilon'\,\Delta l$$

$$\frac{-dI}{I} = C\,\varepsilon'\,dl$$

$$\int_{I_o}^{I} \frac{-dI}{I} = \int_{0}^{l} C\,\varepsilon'\,dl$$

$$\ln \frac{I_o}{I} = C\,\varepsilon'\,l$$

$$\log \frac{I_o}{I} = C\,\varepsilon\,l$$

$$A(\lambda) = \log \frac{I_o}{I} = C\,\varepsilon(\lambda)\,l$$

ε, extinction coeff units ($M^{-1}\,cm^{-1}$, $(mg/ml)^{-1}\,cm^{-1}$)

Note that ε is a function of wavelength. ε(λ).

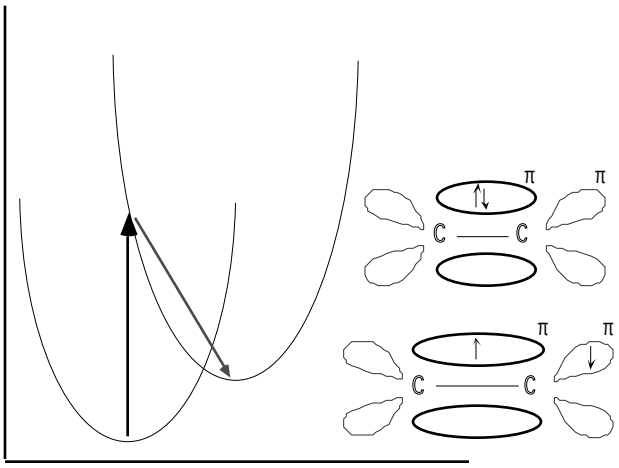
Always pay attention to units!! Always specify units, but when others don't, assume $M^{-1}\,cm^{-1}$.

Electronic transitions - geometry of states and the time scale of the transition

Idea of two states - "molecular geometry" axis

molecular geometry generally is the positions of all atoms and the spin states of the nuclei and electrons.

More typically, one aspect dominates a given transition. For example, in ethylene, the molecular geometry might be simplified to the carbon-carbon internuclear distance. For the case in which the π bond is fully occupied, there is a preferred internuclear separation (short - double bond). For the case in which one electron has been promoted to the π^* orbital, the preferred internuclear separation will be larger (longer - one and one half bond).



Molecular Geometry

Frank-Condon/Born-Oppenheimer: electrons move much faster than nuclei. Nuclei do not move during an electronic transition. They *subsequently* relax to preferred position. The arrows above indicate that the molecule *first* absorbs a photon of light to promote the electron, *after* which the nuclei move to the preferred configuration.

Vibration and rotation are included in the precise definition of the molecular "state"

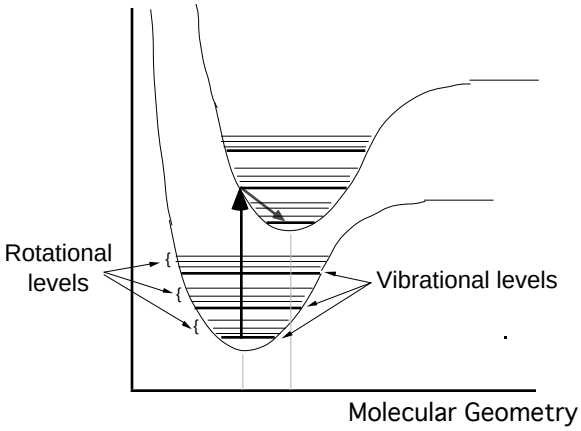
Vibrational sublevels (quantized) exist for each electronic state.

Rotational sublevels (quantized) exist for each vibrational state.

Excited vibrational states are usually not well-populated in the ground state, but transitions to excited vibrational levels within the excited electron states are likely.

Excited ground state rotational levels *are* often significantly populated, but...

Normally the linewidth of the electronic transition due to the inherent heterogeneity of the states makes resolution of vibronic and rotational levels impossible. In these cases, the sublevels are ignored and we speak of the system in electronic terms only.



Molecular Geometry

Particle in a box: energy levels in delocalized systems

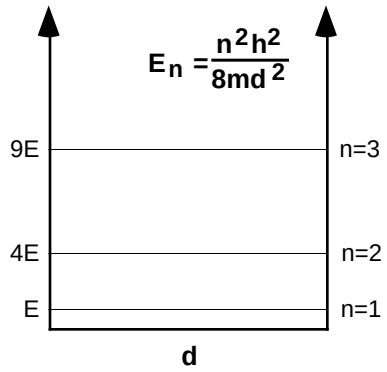
(Not in book...)

$$\mathbf{E_n = \frac{n^2h^2}{8m_ed^2}} \quad \mathbf{n=1,2,3,4...}$$ (m_e = mass of the electron)

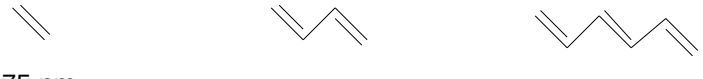
Note that the size of energy gap between the levels is inversely proportional to the square of the size of the box, d.

Also note to always count the total number of electrons and fill the lowest levels first. The lowest energy transition will then be from the highest occupied orbital (HOMO) to the lowest unoccupied orbital (LUMO).

Back to ethylene. Expansion of conjugated system



lowers energy, as predicted.

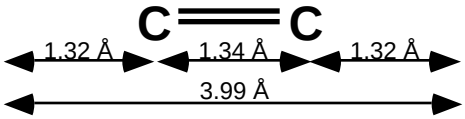


175 nm
ε=15,000 M^{-1} cm^{-1}

217 nm
ε=21,000 M^{-1} cm^{-1}

258 nm
ε=35,000 M^{-1} cm^{-1}

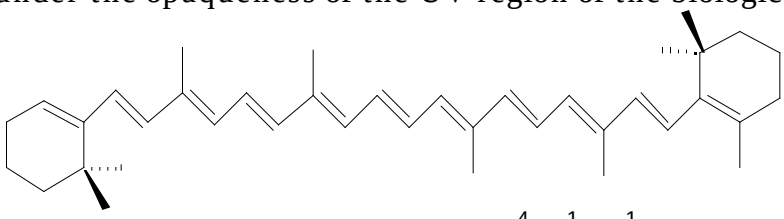
Using the equation above, we can calculate the size of the "box" for the case of ethylene. Noting that each level can contain two electrons (Pauli exclusion principle), we find that the n=1 state is fully populated and that the lowest energy transition will be from n=1 to n=2. The difference in energy, $E_2 - E_1$, can be obtained from the observed wavelength of the transition, finally yielding d = 3.99 Å.



Since we know that the carbon-carbon distance for a double bond is 1.34 Å, the box must extend 1.32 Å on either side.

distances for other boxes are 4.44 Å and 4.84 Å.

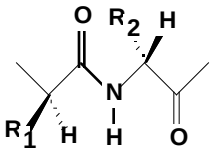
There are many examples in biology of large π systems: nucleic acid bases, porphyrins, visual and pigment chromophores, ... Building a "bigger box" is nature's way of bringing absorptions into the "visual" region of the spectrum, or rather, of bringing "vision" out from under the opaqueness of the UV region of the biological spectrum.



452 nm ε = 15.2 x 10^4 M^1 cm^{-1}

β-carotene (found in carrots)

The peptide bond can also be thought of as a "box." Note that there is a π system extending from the carbonyl oxygen, through its carbon, and across to nitrogen. Delocalization onto the electronegative oxygen and into its and nitrogen's lone pairs, makes the box bigger than you might first think. Note that there are two transitions in the UV region, but that one is much more allowed than the other. The absorption at 190 nm is more commonly monitored, although it is getting very close to the vacuum region of the UV where everything absorbs.

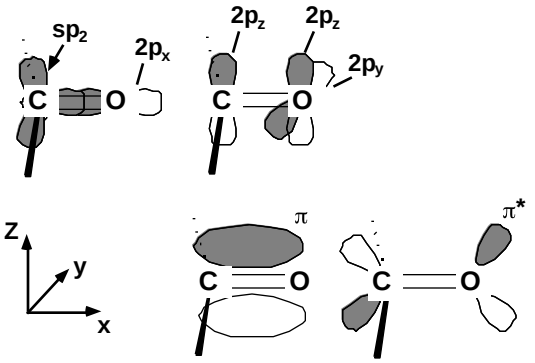


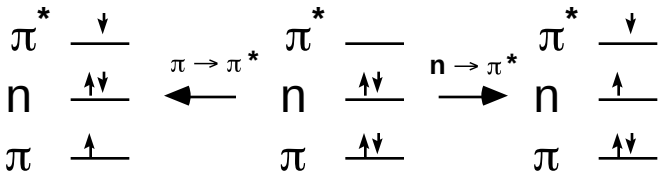
Peptide Bond
$\pi \rightarrow \pi^*$ 190 nm $\varepsilon \approx$7,000 M^{-1} cm^{-1}
$n \rightarrow \pi^*$ 220 nm $\varepsilon \approx$200 M^{-1} cm^{-1}

We now have a qualitative feel for transition <u>energies</u> (the wavelength of the transition). What about the <u>probability</u> of a transition?

<u>Symmetry dictates transition probabilities</u>: Formaldehyde as a simple model. (CS p. 370)

We've learned about geometrical constraints on transition <u>energies</u> (→ absorption wavelength). What about transition <u>probabilities</u> (→ absorption intensities)?



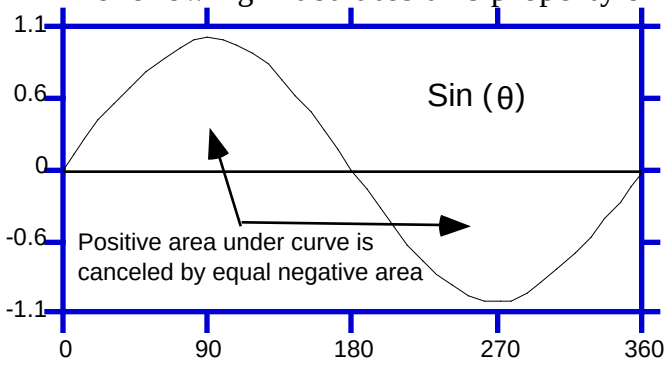


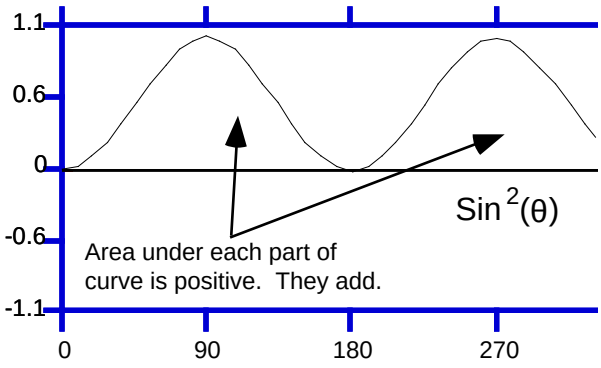
An sp^2 hybridized carbon can combine with a simple unhybridized oxygen to form π and π^* orbitals.

The ground state of formaldehyde can be represented as $n^2\pi^2$ (highest occupied orbitals only).

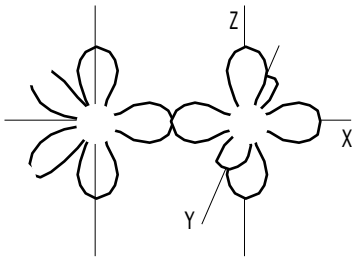
Remember that the formula for the transition probability contains the term $|\langle\psi_b|\boldsymbol{\mu}|\psi_a\rangle$, and note that this term is an <u>integral over all space</u>. If the integrand has odd symmetry, the integral will go to zero, whereas if it has even symmetry, the integral will be non-zero. We can examine the symmetry of each orbital and of each of the electric dipole operators associated with the light.

The following illustrates this property of integration:

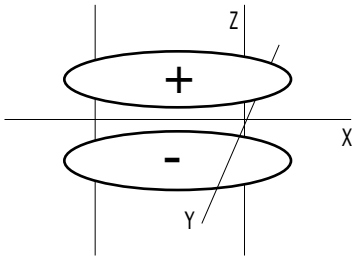
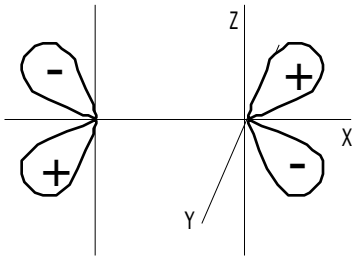
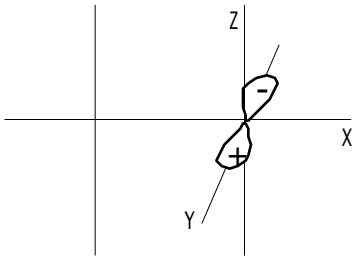
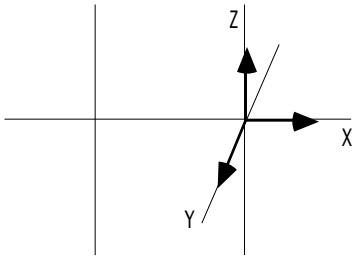




Formaldehyde
CH_2O



Symmetry

		xy	xz	yz	reflected throug
		z	y	x	along axis
π		odd	even	even	
π*		odd	even	odd	
n		even	odd	even	
		even	even	odd	μ_x →
		even	odd	even	μ_y ↘
		odd	even	even	μ_z ↑

$$\langle \psi_i | \boldsymbol{\mu} | \psi_f \rangle = \langle \psi_i | \mu_x | \psi_f \rangle + \langle \psi_i | \mu_y | \psi_f \rangle + \langle \psi_i | \mu_z | \psi_f \rangle$$

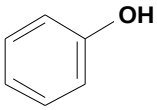
$$\iiint \psi_i \, \mu_x \, \psi_f \, \partial x \partial y \partial z + \iiint \psi_i \, \mu_y \, \psi_f \, \partial x \partial y \partial z + \iiint \psi_i \, \mu_z \, \psi_f \, \partial x \partial y \partial z$$

$\langle \pi \| \boldsymbol{\mu} \| \pi^* \rangle$	$= \langle \pi \| \mu_x \| \pi^* \rangle$	$+ \langle \pi \| \mu_y \| \pi^* \rangle$	$+ \langle \pi \| \mu_z \| \pi^* \rangle$
	[(oeo)(eee)(eoo)]	+ [(oeo)(e$\not{o}^{0}$e)(e$\not{e}^{0}$o)]	+ [(o$\not{o}^{0}$o)(eee)(e$\not{e}^{0}$o)]
	non-zero	+ 0	+ 0
	$\neq 0$ **(allowed)**		
$\langle n \| \boldsymbol{\mu} \| \pi^* \rangle$	$= \langle n \| \mu_x \| \pi^* \rangle$	$+ \langle n \| \mu_y \| \pi^* \rangle$	$+ \langle n \| \mu_z \| \pi^* \rangle$
	[($\not{e}^{0}$eo)(o$\not{e}^{0}$e)(eoo)]	+ [[($\not{e}^{0}$eo)(ooe)(e$\not{e}^{0}$o)]	+ [(eoo)(o$\not{e}^{0}$e)(e$\not{e}^{0}$o)]
	0	+ 0	+ 0
	$= 0$ **(forbidden)**		

Aromatic groups can be more complicated, but nevertheless, symmetry is often very important. Extinction coefficient depends on symmetry. Benzene is "too symmetrical." A dipole does not exist and is not easily induced. Consequently the transition probability (and therefore the extinction coefficient) is very small.

Benzene

256 nm $\varepsilon = 400\ M^{-1}\ cm^{-1}$

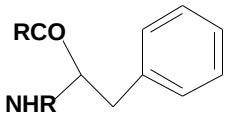


Phenol

271 nm $\varepsilon = 2{,}000\ M^{-1}\ cm^{-1}$

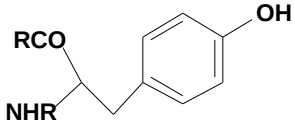
Introduction of the hydroxyl group in phenol, breaks the symmetry in one plane, producing a dipole in the molecule. The extinction goes up dramatically.

The amino acids phenylalanine and tyrosine are analogous to benzene and phenol. The effects of symmetry are similarly illustrated.



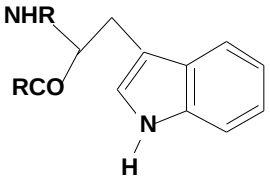
Phenylalanine

250 nm $\varepsilon = 200\ M^{-1}\ cm^{-1}$



Tyrosine

274 nm $\varepsilon = 1{,}400\ M^{-1}\ cm^{-1}$



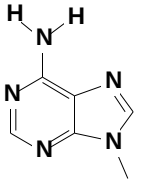
Tryptophan

3 transitions 240-290 nm

$\varepsilon_{280} = 5{,}700\ M^{-1}\ cm^{-1}$

Tryptophan is more complex. In particular, there are three transitions in the region of the UV spectrum from 240 to 290, each with its own characteristic extinction. The net absorption "band" represents the sum of these transitions. The quoted extinction coefficient does not reflect one single transition.

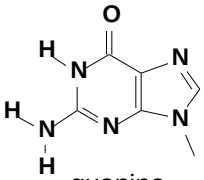
Nucleic acids are similarly "big boxes." The spectra of several nucleotides actually show the composite nature of the absorption band, as shoulders on the main envelope can be seen. The sugar and phosphate components have negligible effect, but base-base interactions can have significant effects (we'll see later).



adenine

259 nm

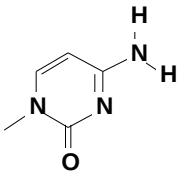
ε = 15,400 M^{-1} cm^{-1}



guanine

253 nm

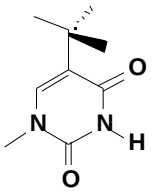
ε = 13,700 M^{-1} cm^{-1}



cytidine

271 nm

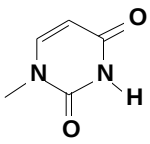
ε = 9,000 M^{-1} cm^{-1}



thymidine

267 nm

ε = 9,600 M^{-1} cm^{-1}



uracil

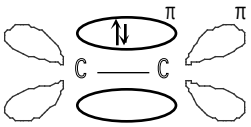
262 nm

ε = 10,000 M^{-1} cm^{-1}

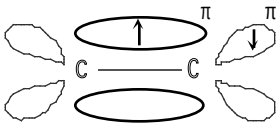
Structure from spectroscopy - whole not the sum of the parts.

Solvent effects

If you know something about the nature of a transition, you can often predict the effect of solvent on the absorption properties of a chromophore. In general, solvent interactions <u>stabilize both the excited and the ground</u> states. If this effect is equal for the two, the energy of the transition will not change.



1

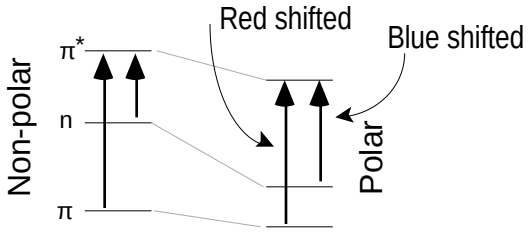


2

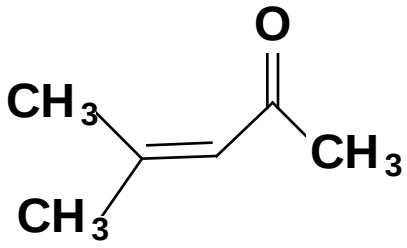
<u>Solvent polarizability</u> is important. If a particular electronic state is characterized by a large dipole moment, then a highly polarizable solvent will stabilize that state more than a less polarizable solvent. **N.B.**

Note in the structures at right for ethylene: the π^* orbital is more diffuse than the π orbital. Consequently it will have a larger inducible dipole moment. The π^* orbital will be stabilized more than the π orbital by interaction with a polarizable solvent.

Example: mesityl oxide (CS p. 387).



Solvent	$\pi \rightarrow \pi^*$ λ_{max}	$n \rightarrow \pi^*$ λ_{max} (nm)
Hexane	230	327
Ethanol	237	315
Water	245	305



(Note: the book is wrong!)

As for formaldehyde, 3 energy levels dominate the low energy transitions, yielding $\pi \rightarrow \pi^*$ and $n \rightarrow \pi^*$ transitions. Look at solvent polarizability.

The electron in the <u>π orbital</u> is localized primarily between the involved nuclei.

In contrast, the <u>π^* orbital</u> is more diffuse and extends well beyond the central nuclei, consequently one expects the π^* orbital to be influenced much more by solvent than the π orbital. In particular, a more polar solvent should stabilize the dipole in the π^* orbital much more than it stabilizes the π orbital - this leads to a reduction in their energy separation so that the $\pi \rightarrow \pi^*$ absorption will shift to lower energy (longer wavelengths - to the red) in a more polar solvent.

An electron in a <u>non-bonding orbital</u> (for the simplest case, a lone pair) is readily available for interactions with solvent (*e.g.* as a H-bond acceptor). Therefore we expect the shift to be even larger for the $n \rightarrow \pi^*$, however in this case the ground state is stabilized relative to the excited state, so that the difference in energy between the two states will *increase* (the transition will move to lower wavelength - a blue shift).

Solvent perturbation spectroscopy.

In limited cases, one can monitor <u>exposed</u> surface amino acids by their response to changing solvent. Amino acids such as tyrosine and tryptophan which are exposed to solvent will show well characterized shifts as the polarity of the solvent is reduced (by addition of alcohol for example). In

contrast, buried residues may be relatively insulated from changes in the solvent. In ideal cases, this can be used to determine the number of each type of aromatic residue exposed to solvent.

More interestingly, if a protein undergoes a major conformational reorganization, previously buried groups may become exposed to solvent and vice versa. This will be observed as an abrupt change in the spectrum accompanying the conformational change.

An extreme example of this is the change in the exposure of buried aromatic residues accompanying the unfolding of a native protein structure. In fact, this approach is used in many studies of thermal denaturation (although calorimetry provides more information).

The environment in a protein can vary dramatically.

The local environment that a given electron sees is very dependent on the environment and is usually quite *anisotropic* (not homogeneous). By this we mean that polarizability and the distribution of dipoles is not uniformly distributed around a point in space, as in solution. One end of an electronic orbital may be situated near a carbonyl oxygen from the peptide bond, while the other end is in close proximity to a hydrophobic methyl group from alanine. Thus it is very difficult to predict quantitatively the energy levels within a protein from studies of solvent shifts.

Protonation state can significantly effect absorption maxima, extinction coefficients.

Tyrosine has a pK_a of 10.9. Deprotonation leads to a significant shift in the absorption maximum towards the red. This can be exploited to titrate Tyr residues in a protein.

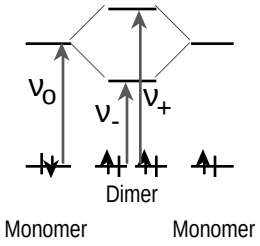
For nucleic acid bases, protonation of C or G leads to large red shifts. Deprotonation of U or T also results in a large red shift. Protonation of A leads to a small change.

Interactions *between* chromophores - the *exciton* effect.

For a theoretical description, see CS 390-395.

What happens when we have two identical chromophores? For two non-interacting monomeric chromophores, the result is intuitively obvious - the resulting spectrum is that of the monomer, but with twice the intensity.

For two *interacting* monomers, we again note that in general the ground states of a molecule are less polar than the excited states. Consequently excited states of molecules can interact with each other through dipolar interactions, in the same way that the excited state of a monomer interacts with polar solvent. The result is that the energies of the ground states for the monomers are effected much less by their interaction than are the energies of their excited states.



Combining the excited states in much the same way as one combines atomic orbitals to form molecular orbitals, we see that the two resulting excited states are equally higher and lower in energy than the excited state of the non-interacting monomer. In equation form:

$\nu_+ = \nu_o + V_{12}$ $\nu_- = \nu_o - V_{12}$ $2V_{12}$ is called the exciton splitting.

The interaction energy V_{12} depends on various factors, but to a first approximation falls of as R_{12}^3 (that is, it falls off rapidly with distance).

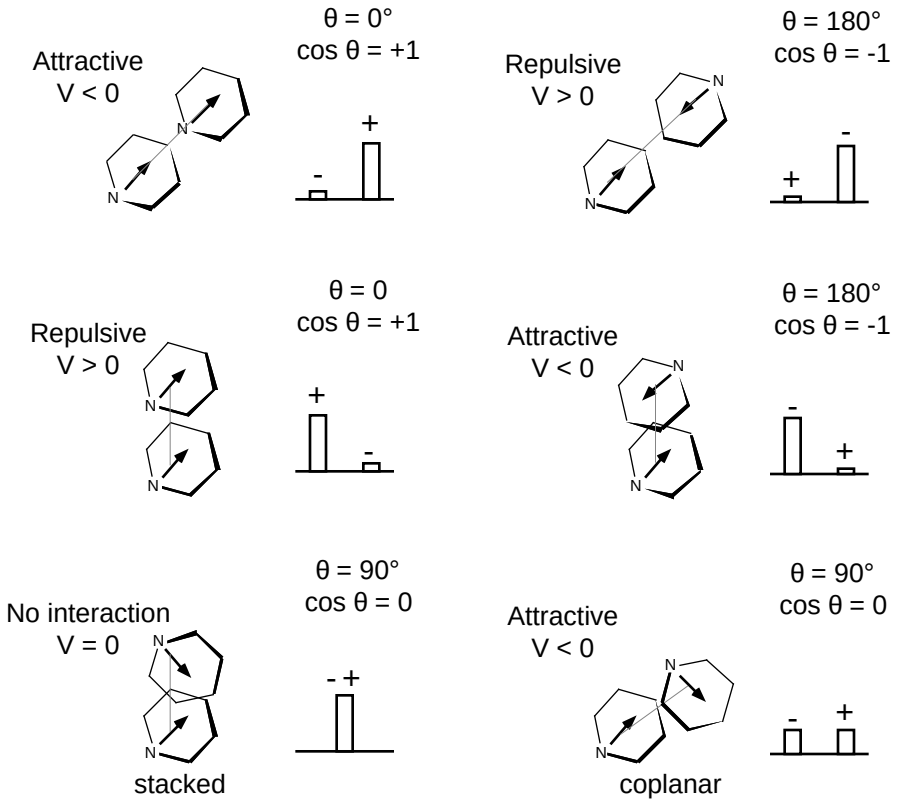
Similarly, it can be shown that the relative intensity of each transition is split about the original intensity (*i.e.* $I_+ + I_- = 2I_o$), but their relative intensities depend on the angle between the two transition dipoles, θ

$D_\pm = D_o \pm D_o \cos\theta$ See page 396 in CS for a description of this effect.

Interactions between identical dimers.

The spectrum of two *identical* and *non-interacting* chromophores is simply the spectrum of one, but with twice the intensity.

We saw above that the ground and excited states of different molecules can interact with each other. What happens when two identical chromophores interact? One important feature of such an interaction can be seen when we consider the transition dipole vector of each chromophore. The interaction has a strong angular dependence (the dipole-dipole interaction).



The intensities and the splittings depend on the angle θ; the intensity of the resulting band is proportional to the magnitude of the vector resulting from the addition (or subtraction) of the contributing transition dipole vectors.

So we see that chromophores can interact positively or negatively, resulting in split transitions, not necessarily of the same intensity.

Extension to systems of more than two chromophores.

By analogy, we see that 3 interacting monomers will give rise to 3 absorption bands, and so on for larger groups of interactions. What happens for a very long polymer? For a regular structure such as an α-helix, symmetry considerations require that almost all of the bands will have zero intensity. Only two allowed $\pi \rightarrow \pi^*$ transitions remain: 1 parallel to the helix axis and 1 perpendicular.

Example: (CS p. 369) oriented poly-L-glutamic acid film. The molecule forms long α-helices which align parallel to one another. Measurement of absorption spectra using light polarized parallel or perpendicular to the helix axis shows distinctly different absorption maxima and extinction coefficients.

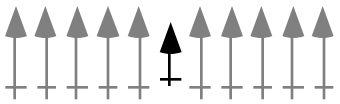
Hypochromism (*less color*): interaction between *different* transitions in different molecules.

Simplistically, for a long polymer of interacting monomers, the overall integrated absorption intensity should be the sum of that of the individual monomers. This is often not true, as displayed in DNA. The intact double helix absorbs 30% less than a mixture of the same monomers. The best explanation of the effect, by Tinoco and Rhodes, is (according to CS) "not simple."

So far, when looking at monomer-monomer interactions, we have considered interactions between two identical transitions within each monomer. But a transition in one monomer can be influenced by ***other*** transitions in its neighbor.

Very simplistically, we can see the effect as the effects of induced dipoles. Remember that an electric field will induce a dipole in each orbital's electronic clouds. If such dipoles are all aligned parallel to each other as shown in the first case below, there will be a mutual repulsion. This makes the induction of each dipole more difficult.

Hypochromism



Hyperchromism

Similarly, if the dipoles are aligned as in the lower figure, there will be a mutual attraction, making the induction of dipoles easier. We can now see that in the first case, the transition probability will be reduced (extinction coefficient smaller - hypo (under)), while in the second case it will be increased (extinction coefficient larger - hyper (over)),.

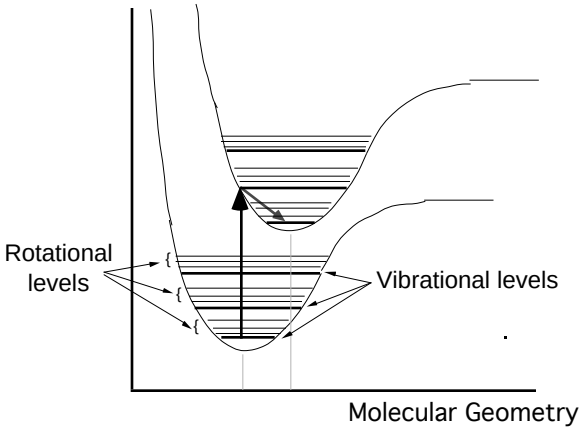
Since this is a dipole-dipole interaction, the strength of the interaction goes as $1/r^3$. The chromophores must be relatively close in space. As we saw above, the angle of the chromophores' transition dipoles is also critical. It also requires a polarizability in both molecules.

The former case (hypochromism) occurs for duplexes of DNA, hence we observe a hypochromic decrease in the extinction coefficient for duplex DNA vs. melted DNA (or the component free nucleotides).

Fluorescence and Phosphorescence Spectroscopies

Introduction - emissive transitions

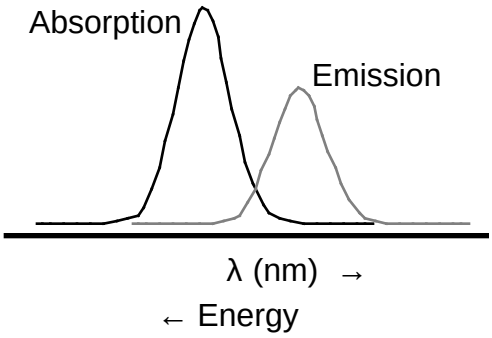
Review: We saw before that when a molecule in the ground vibronic level of the ground state is influenced by light of the appropriate energy, transitions are induced between that state and higher lying excited electronic states. We also saw that during the time course of the electronic excitation, nuclei don't move appreciably. Consequently excitation is often to excited vibronic and rotational levels within the molecule.



Non-radiative and radiative processes

The excitation from S_o to S_1 is a radiative absorption. Radiative: involving light. Subsequent to this excitation, the molecule relaxes to the ground vibronic (and rotational) sublevels within the excited electronic state. This process is *non-radiative*, that is, it is not associated with light energy, but rather represents a transfer of thermal energy to the surroundings. For example, a diatomic molecule in an excited vibrational state may collide with a solvent molecule and transfer that vibrational energy to the solvent molecule.

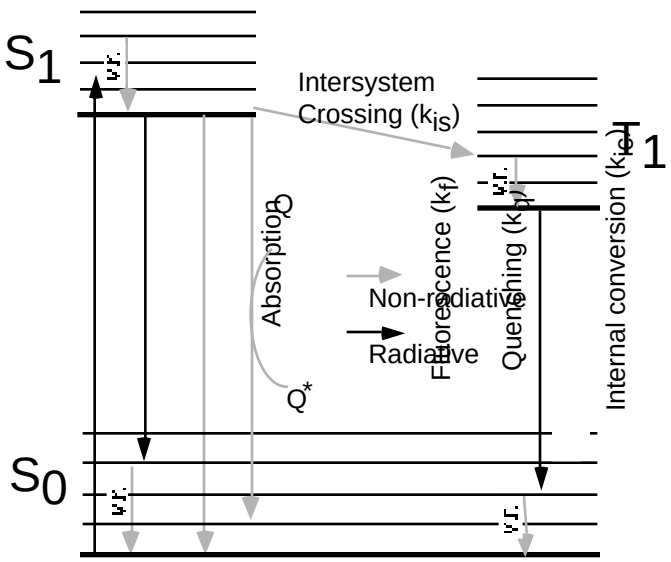
The resulting molecule, in its ground vibronic sublevel within the excited electronic level, may now emit a photon of light and *spontaneously* revert to the ground electronic state S_o (**fluorescence** - a radiative emission process). But again, the electronic transition is faster than the movement of nuclei, so that it relaxes to an excited vibronic sublevel of the ground electronic state. Consequently, light emitted in fluorescence is always of lower energy than that originally absorbed.



Note that there exist pathways for *non-radiative* transitions between electronic energy levels. It is due to these processes that we can rarely excite enough molecules between states (with illumination at the transition energy) that we equalize the populations in the two electronic states. In fact, with most spectroscopies, the populations of the individual levels remain very close to their Boltzmann distributions, despite our

induction of transitions between states. Non-radiative pathways predominate over radiative ones.

Another non-radiative process which can occur is called inter-system crossing. In this process, the singlet excited state is converted into a triplet. Remember that during the initial absorption transition, we took an electron from a fully occupied and therefore spin-paired level (a singlet level). During that transition the spin does not change, so that the resulting excited state is still a singlet. However, in this excited state, the two electrons are in singly occupied orbitals. We know that the triplet state will always be lower in energy (with both spins aligned, we say they are more correlated and stay away from each other better - see an introductory quantum mechanics text).



Such a transition, however, is formally forbidden (remember that there must be a mechanism to induce the transition), so that the rate of intersystem crossing is generally very slow. For systems that do make this transition, the relaxation back to the original singlet ground state is also formally forbidden, so that the lifetime of the triplet state can be much longer than that of the corresponding excited singlet state. The radiative transition back to the singlet ground state is called **phosphorescence**. In the case that non-radiative pathways between electronic states (internal conversion and intersystem crossing) are slow relative to the rate of spontaneous emission, fluorescence and/or phosphorescence can be observed.

Radiative processes summary:

absorption - as discussed previously

fluorescence - transition back to the ground electronic state, with emission of a photon of the corresponding energy.

phosphorescence - transition from an excited **triplet** back down to the ground state **singlet**, with emission of a photon of the corresponding energy. This transition is formally forbidden, so that for most systems the rate of this transition is very low - the triplet state once generated has a much longer life time in general than does the excited singlet state.

Non-radiative processes summary:

vibrational relaxation (v.r.): transitions with the vibronic sublevels of an electronic state. The transition energies are usually near kT for room temperature. The rate of such transitions is generally very high ($t_{1/2} = 10^{-12}$ sec).

internal conversion: occurring at a rate k_{ic}. The associated energy released is transmitted to the environment via non-radiative energy transfer, such as collision with solvent or with other molecules. In general k_{ic} will increase with temperature (at the expense of other transitions, such as fluorescence and phosphorescence).

intersystem crossing: in this case a formally *forbidden* "spin flip" converts the system from a singlet state (all electrons paired) to a triplet (a system with a net electron spin of 1). Note that direct excitation from the ground state singlet to the excited state triplet is strongly forbidden.

quenching: like internal conversion, this processes often derives from processes such as molecular collisions (or chemical reaction), but in this case the energy transfer is large enough to lead to transitions between *electronic* states of the molecule. See below for more.

Excited Electronic State Lifetimes

Singlet excited state (S_1): Generally the rate of spontaneous emission from this state is fast. Consequently, the lifetime of this state is very short (typically $t_{1/2} = 10^{-12}$ sec).

Triplet excited state (T_1): Since the transitions back down to the singlet ground state are formally forbidden, the lifetime of this state is significantly longer than rates for transitions from singlet to singlet).

Quantum yield

Each process of "deexcitation" has associated with it a rate constant. The relative yield of each pathway depends critically on the rates of each of these processes. The quantum yield for a particular pathway is defined as the ratio of the rate of that pathway to the sum of the rates for all pathways from that state (or, alternatively, the denominator can be expressed as the rate of excitation to that state).

$$\varphi_i = \frac{k_i}{\sum_{j=1}^{n} k_j} = \frac{k_i}{k_{\text{all dexcitation}}} = \frac{k_i}{k_{\text{excitation}}}$$

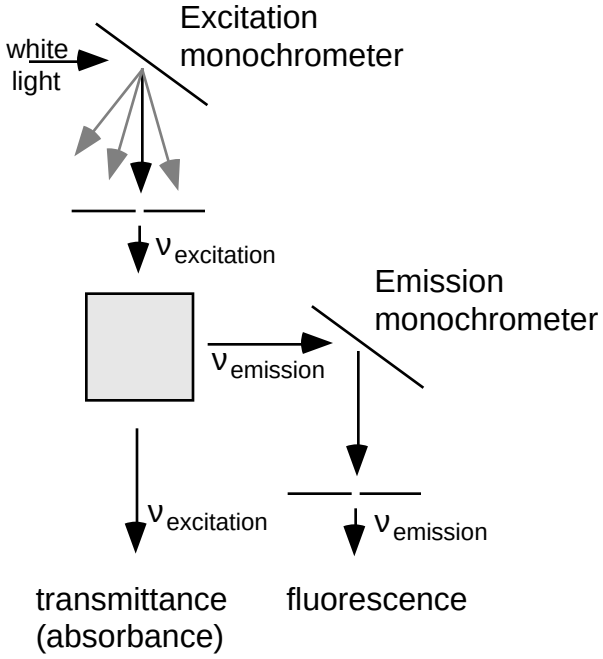
In other words, the quantum yield for fluorescence is the ratio of the photons emitted in fluorescence to the photons absorbed by the original transition.

$$\varphi_F = \frac{k_F}{k_F + k_{ic} + k_{is} + k_q[Q]} = \frac{\text{deexcitation via fluorescence}}{\text{all deexcitation}} = \frac{\text{photons emitted}}{\text{photons absorbed}}$$

It is the latter description of quantum yield that is typically measured experimentally.

The fluorescence experiment

Experimentally, fluorescence is a **very sensitive** technique. In absorption, we detect the light which passes through the sample. In a very dilute solution, we may have 0.01% of the light absorbed, such that the light that passes through may represent 99.99% of the incident light. Consequently, the absorption measurement is a very small difference of two much larger numbers. In fluorescence, we directly detect the emitted photons in the absence of any other light. This is a much more sensitive measurement.



In the fluorescence experiment we can record both the absorption spectrum and the emission spectrum. In both cases, we measure the emitted light.

1) **Fluorescence excitation**. In the first case, we set the emission monochrometer to the wavelength of the fluorescence emission maximum and scan the excitation monochrometer.

2) **Fluorescence emission**. In the second case, we excite with only light corresponding to the maximum excitation, and then we scan the emission monochrometer.

In practice, for an unknown sample, one must iterate back and forth between these approaches to determine the maxima for both excitation and emission.

Within an electronic transition, the largest signal will generally be lowest in energy and will correspond to transitions from the lowest vibronic level of the ground state (excitation) to the lowest vibronic level of the excited electronic state.

Internal Conversion

In internal conversion, a molecule in an excited vibrational state can collide with a solvent molecule, transferring its energy to the solvent molecule and reverting to a lower energy vibronic level. The solvent molecule gains kinetic energy (it is "heated").

Quenching via Chemistry

The energy difference between the ground and excited states (the excitation energy) can be used to perform chemistry. A second molecule can interact with the excited state of the absorber, so as to allow relaxation of the absorber coupled to the input energy into a chemical reaction involving the quencher. This type of reaction will generally show a dependence on the concentration of the quenching molecule (as well as the absorber). Studying the concentration dependence of the quenching can provide support for and further information on the quenching mechanism.

A variation of quenching is direct chemistry involving the absorber. The excited state of a molecule is chemically different, often being characterized by weakened bond character. As an example, in ethylene the $\pi \rightarrow \pi^*$ transition takes a system with a double bond between carbon, and converts it to a system with a net single bond between carbons. In more complex systems, if other stresses pre-exist which strain that single bond, the reduction in bonding associated with the $\pi \rightarrow \pi^*$ transition might lead to bond cleavage.

It is this mechanism which is usually exploited in "photo-bleaching." The product of the chemical reaction no longer has the absorption properties of the original "reactant." We will mention this later.

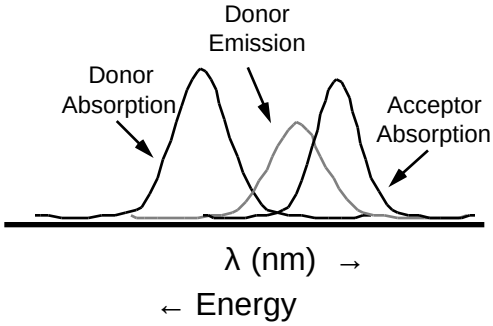
Transfer of excitation energy

Finally, a molecule in an excited electronic state can transfer its excitation energy to another molecule. In the process, the donor molecule relaxes from the its excited to its ground electronic state, while the acceptor molecule is excited from the ground to its excited electronic state. We can write the efficiency of transfer as

$$E = \frac{k_T}{k_T + k_F + k_{ic} + k_{is}} \quad \text{where } k_F, k_{ic}, \text{ and } k_{is} \text{ are properties of the donor.}$$

For appreciable transfer of energy, two requirements hold:

1) A mechanism for transfer must exist.
   Typically, a dipole-dipole interaction can lead to a coupled transition between electronic states. The dipole of the donor's excited state can interact with the ground state of the absorber in a manner similar to the interaction of light's electric field with the ground state of the absorber. In the quenching pathway of energy transfer, the donor is de-excited to the lower electronic state, while the acceptor is excited to its higher excited electronic state.
2) The involved energies must match (as for light (**E**) induced transitions).
   Re-stated: there must be an appreciable overlap of the fluorescence spectrum of the donor and the absorption spectrum of the acceptor.



A quantitative theory for such energy transfer has been developed by T. Förster (see CS 451-453). In this theory, the rate of energy transfer is given by

$$k_T = \frac{1}{\tau_D}\left(\frac{R_o}{R}\right)^6 \qquad \tau_D = \frac{1}{k_F + k_{ic} + k_{is}}$$

NOTE: Cantor & Schimmel is ***wrong*** for k_T!!

where τ_D is the lifetime of the donor in the absence of the acceptor, R is the distance between donor and acceptor.

R_o is a factor called the "characteristic transfer distance." It contains contributions from

1) the spectral overlap between donor and acceptor (energies match)
2) the refractive index of the medium between the two
3) the orientation of the two with respect to each other - dipoles must be able to interact with each other (this component can average out for rapidly tumbling molecules).

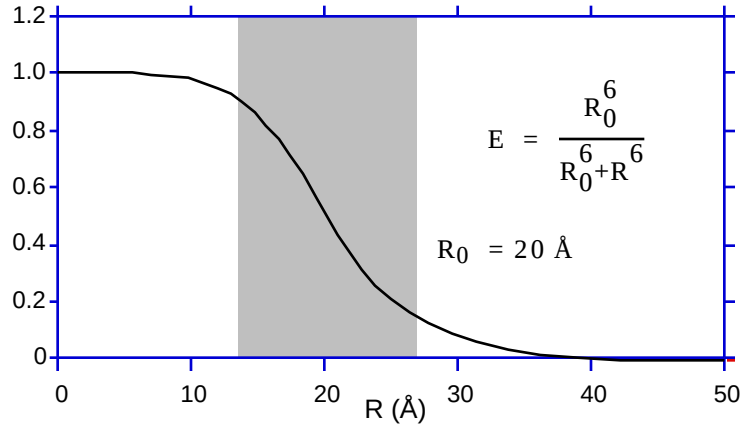
Note that the distance, R, comes into this equation as the inverse of the 6th power. This is due to the fact that coupling mechanism is a dipole-dipole interaction. The leading

term in the dipole-dipole interaction is dependent on R^3, and this term is squared in computing the transition probability.

If we rewrite the efficiency of energy transfer as $E = \frac{k_T}{k_T + 1/\tau_D}$

then substituting for k_T from before:

and substitute in for k_T, then we have $E = \frac{R_0^6}{R_0^6+R^6}$



It can be seen that this is most sensitive for distances R near R_o. Values of R_o typically range from 10 Å to >50 Å. Distances between chromophores of as much as 80 Å between chromophores have been measured.

More thorough explanation:

The transition we are looking for is $\psi_{Do}\psi_{A1} \rightarrow \psi_{D1}\psi_{Ao}$ (where 0 refers to ground state and 1 to the excited state) and arises through a dipolar interaction between donor and acceptor. The probability of this transition is proportional to

$$Rate \quad \left| \left\langle \Psi_{D_0}\Psi_{A_1} \left| \frac{\tilde{\mu}_D \ \tilde{\mu}_A}{R^3} - \frac{3\left(\tilde{\mu}_D \ \tilde{R}\right)\left(\tilde{R} \ \tilde{\mu}_A\right)}{R^5} \right| \Psi_{D_1}\Psi_{A_0} \right\rangle \right|^2$$

Assume that one can separate out the angular dependence of the dipolar term as below:

$$\frac{\tilde{\mu}_D \ \tilde{\mu}_A}{R^3} - \frac{3\left(\tilde{\mu}_D \ \tilde{R}\right)\left(\tilde{R} \ \tilde{\mu}_A\right)}{R^5} = \frac{(\cos\theta_{DA})\mu_D\mu_A}{R^3} - \frac{3(\cos\theta_D)(\mu_D R)(\cos\theta_A)(R\mu_A)}{R^5}$$

$$= (\cos\theta_{DA})\frac{\mu_D\mu_A}{R^3} - 3(\cos\theta_D)(\cos\theta_A)\frac{(\mu_D R)(R\mu_A)}{R^5}$$

$$= \left[\cos\theta_{DA} - 3\cos\theta_D \cos\theta_A\right]\frac{\mu_D\mu_A}{R^3} = \kappa\frac{\mu_D\mu_A}{R^3}$$

$$Rate \quad \left| \left\langle \Psi_{D_0}\Psi_{A_1} \left| \kappa\frac{\mu_D\mu_A}{R^3} \right| \Psi_{D_1}\Psi_{A_0} \right\rangle \right|^2 = \frac{\kappa}{R^6}\left| \left\langle \Psi_{D_0}\Psi_{A_1} | \mu_D\mu_A | \Psi_{D_1}\Psi_{A_0} \right\rangle \right|^2$$

$$= \frac{\kappa}{R^6}\left| \left\langle \Psi_{D_0} | \mu_D | \Psi_{D_1} \right\rangle \right|^2 \left| \left\langle \Psi_{A_1} | \mu_A | \Psi_{A_0} \right\rangle \right|^2$$

Caveats... Note that in a complicated system such as biology often throws at us, there may be multiple chromophores present. If the absorption maximum of one is close to the emission maximum of the other, the former can absorb the light emitted by the latter. This is normally a problem only when the absorber is present at high concentrations and/or has a very large extinction coefficient. This is a fundamentally different process than that seen above, since it involves no direct interaction between the two molecules. In this case the fluorophore emits photons independent of the

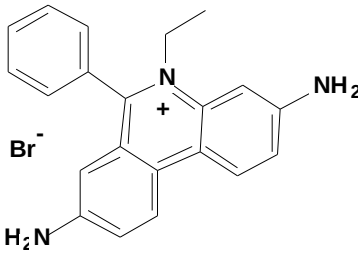
acceptor. The absorbing molecule may, of course, not fluoresce. This latter situation is rare.

If we examine the experimental design for fluorescence spectroscopy presented above, we can see that if the fluorescent molecule absorbs light VERY strongly, then a large percentage of the excitation light may be absorbed in that part of the cuvette very near the source. If your fluorescence detection is focused towards the center of the cuvette, you may be looking at molecules which are in the "shadow" of the molecules near the source. This is called the "inner filter effect." One should always check the absorption spectrum of the sample to see that absorption is not too large.

Fluorescence and the Environment

Just as we saw in absorbance, the environment of a fluorophore can have dramatic effects on the electronic states of the molecule, and hence on its fluorescence properties. Stabilizing and destabilizing interactions with excited and ground state wavefunctions can alter not only the energies of the transitions, but also the rates of each of the radiative and non-radiative processes. We have seen, for example, how neighboring groups can quench or transfer fluorescence through a dipole-dipole mechanism. Although the absorption process for any given moleculre is complete within 10^{-15} sec (remember to distinguish this from the rate at which photons are absorbed in a *bulk* solution, probability), there is often an appreciable delay between absorption and fluorescence - generally 10^{-9} to 10^{-8} sec. During this time, other process can occur: collisional quenching as we've just seen, protonation/deprotonation, local conformational changes, etc.

Many fluorescent molecules, such as ethidium bromide (EtBr), undergo rapid solvent quenching of their excited singlet states. Consequently, EtBr in water does not have a large quantum yield for fluorescence in solution. However, when it intercalates into DNA, it not only interacts with the DNA base π systems, but also is relatively protected from solvent. The result is that the fluorescence quantum yield goes up significantly. This is the basis of EtBr staining of duplex DNA in gels.



Fluorescent probes: many of the common "Bio-Absorbers" are only weakly fluorescent if at all (see p. 443 CS). This can work to our advantage. In many cases, one can introduce a fluorescent probe into one's system, for example bound near the active site of an enzyme.

Time-dependent measurement of fluorescence.

For the processes discussed above, we can write:

$$\tau_R = \frac{1}{k_F} \qquad \tau_F = \frac{1}{k_F + k_{ic} + k_{is} + k_q(Q)} \qquad \varphi_F = \frac{\tau_F}{\tau_R}$$

Where τ_R is termed the "radiative lifetime" and τ_F the "fluorescence lifetime." Note that $\tau_F < \tau_R$. The radiative lifetime is related simply to the probability of a molecule in the excited state emitting a photon. The fluorescence lifetime represents the lifetime of the excited state as a result of *all* de-excitation processes (radiative and nonradiative).

So far we have discussed only "steady-state" measurements of fluorescence. Modern spectroscopic techniques now allow us to look at fluorescence in real time. We can, for example, excite a chromophore with a very short (1 nsec), intense pulse of light and then "watch" the fluorescence decay slowly away as the excited state becomes depopulated. If we call $S_b(t)$ the concentration of excited state singlets, we can write:

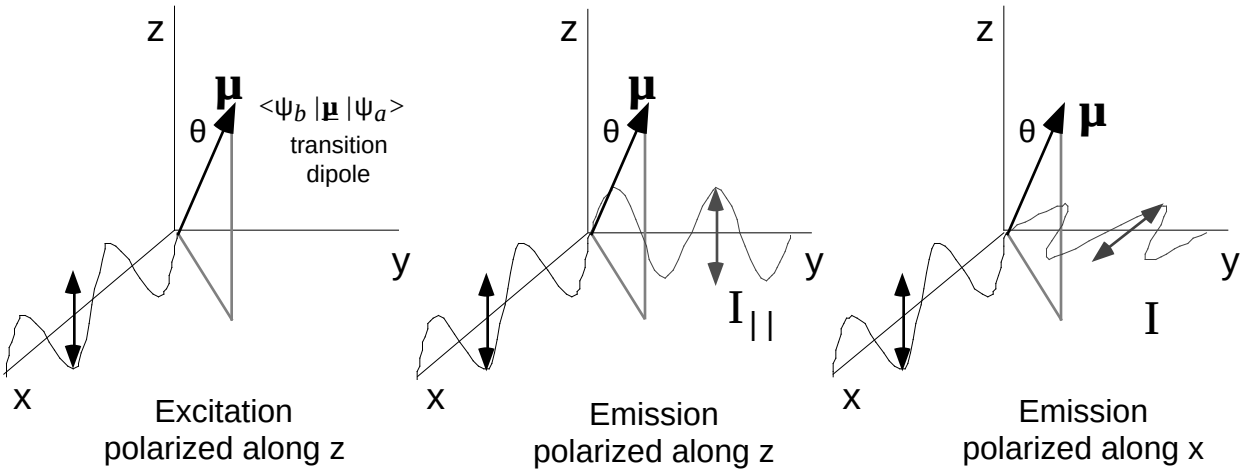
$$\frac{-d(S_b)}{dt} = [k_F + k_{ic} + k_{is} + k_q(Q))]\,(S_b) = \frac{1}{\tau_F}(S_b) \quad \text{(compare with } V = k\,[C]\text{)}$$

$$S_b(t) = S_b(0)\, e^{-t/\tau_F} \quad \text{(integrated as for 1st order kinetics)}$$

$I(t) \quad k_F S_b(t) = k_F S_b(0) e^{-t/\tau_F}$

So that, as expected, the decay follows a simple exponential. One distinct advantage of time-resolved fluorescence spectroscopy is that multiple chromophores can often be distinguished. Two fluorophores with different values of τ_F will produce a decay curve consisting of two exponentials. Curve fitting can resolve these components. This would not be possible in a steady-state measurement.

Fluorescence Anisotropy



We saw before that molecules can have very different transition probabilities in different directions. The electric field component of the light must be able to induce dipole changes in the electronic orbital being excited.

If we illuminate a molecule with light polarized along the z-axis (the **E** vector oscillating along the z-axis), the probability of excitation is a function of $|\boldsymbol{\mu} \cdot \mathbf{E}|^2$ and so is proportional to $\cos^2\theta$, as shown above. Consequently, only a fraction of the molecules in a randomly oriented solution or powder will be excited - selective excitation. If the sample is rigid, then the emitted fluorescence will come from this sub-population and if the fluorescence is associated with the same transition as the original excitation, then the emitted light will retain this same polarization distribution. Emitted light which retains the original polarization is denoted by $\mathbf{I}_{||}$ (fluorescence intensity parallel to the excitation polarization). No fluorescence will be observed perpendicular, $\mathbf{I}_{\perp}$, to the excitation polarization.

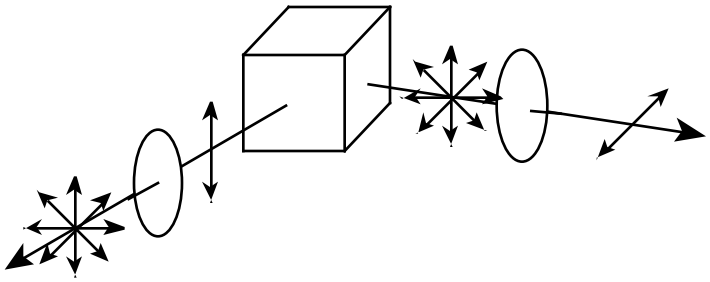
If the lifetime of the excited state is long enough, the molecule will have randomly re-oriented before subsequent fluorescence. The emitted light will have all directions of polarization. The fluorescence intensity will be equal in the parallel ($I_{||}$) and in the perpendicular ($I_{\perp}$) directions.

If re-orientation is comparable to the excited state lifetime, we can define the fluorescence anisotropy as

$$A = \frac{I_{||} - I_{\perp}}{I_{||} + 2I_{\perp}}$$

where again, $I_{||}$ is the intensity of the emitted light observed parallel to the polarization of the excitation light and $I_{\perp}$ is the intensity observed perpendicular.

Examining our two extremes, if reorientation is complete before significant fluorescence, then $I_{||} = I_{\perp}$ and A = 0. If reorientation of the molecule is infinitely slow, then $I_{\perp} = 0$ and A = 1.



Experimentally, we typically start with non-polarized light and pass it through a polarizing filter. To detect light with a particular polarization component, we use a second filter in front of the detector. By rotating these filters with respect to each other, we can determine $I_{||}$ and $I_{\perp}$.

We have assumed that the orientations of the transition moments in the ground and excited electronic states coincide. This is not necessarily the case, such that even in the complete absence of reorientation, a component of the emitted light will be perpendicular to the excitation polarization. In this case, the anisotropy will have a maximum value (A) less than 1. This depolarization is called the *intrinsic* polarization.

In proteins, there are two types of motions to consider.

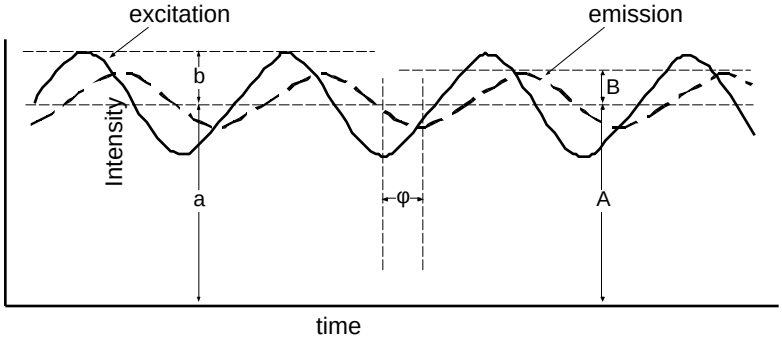
For a fluorophore completely immobilized within a protein, the rotation measured will be the overall rotation of the protein itself.

Rarely is a fluorophore *completely* immobilized within a protein. A tryptophan residue near the surface of the protein will be relatively free to rotate about its axis, even if the rotation of the protein to which it is attached is quite slow. Fluorescence will reflect this local mobility.

Phase Modulated Fluorescence - An alternative to time domain fluorescence decay

The obvious way to measure fluorescence lifetimes is to excited with a brief flash and then watch (in time) the decay of the fluorescence. An alternative, and very useful, approach is a steady state approach which exploits the delay in fluorescence following excitation. If the excitation light is modulated in a sinusoidal fashion, the fluorescence response will reflect this modulation, but as a result of the delay, will be phase shifted (φ) relative to the modulation of the excitation.

The plot below shows an overlay of the excitation and emission profiles:



The phase lifetime is related simply to the phase shift by: $\tan\phi = \omega\tau_p$

where ω is the frequency of the modulation of the excitation.

Remembering that not all excited states deexcite via fluorescence (quantum yield < 1), the intensity of the fluorescence emission will be less than the intensity of the light absorbed. Noting that the modulation of the excitation is not complete, we can define the demodulation factor: $m = \frac{\text{fractional modulation of emission}}{\text{fractional modulation of excitation}} = \frac{\left(B/A\right)}{\left(b/a\right)} = \frac{Ba}{bA}$

with terms as described in the figure above.

The modulation lifetime is related to the demodulation factor by: $m = \frac{1}{\left(1 + \omega^2\tau_m^2\right)^{1/2}}$

For a simple single exponential decay, $\tau = \tau_p = \tau_m$

As an aside, let's derive the above relationships for a single exponential decay:

We can write the time dependent intensity of the excitation light as: $f(t) = a + b\sin(\omega t)$

And note that the fluorescence response will be similar: $N(t) = A + B\sin(\omega t - \phi)$

The time derivative of the response is then: $\frac{\partial N(t)}{\partial t} = B\omega\cos(\omega t - \phi)$

We know that for an instantaneous excitation of a fluorophore, the rate of decay of the excited state population (and therefore of the fluorescence) is a simple exponential with rate constant $k = k_f + k_{ic}$.

$$\frac{\partial N(t)}{\partial t} = -kN(t) = -\frac{1}{\tau}N(t)$$

Adding continuous excitation, we add the intensity of the light

$$\frac{\partial N(t)}{\partial t} = -\frac{1}{\tau}N(t) + f(t) = -\frac{1}{\tau}\left[A + B\sin(\omega t - \phi)\right] + a + b\sin(\omega t)$$

Equating this to the expression earlier, we have:

$$B\omega\cos(\omega t - \phi) = -\frac{1}{\tau}\left[A + B\sin(\omega t - \phi)\right] + a + b\sin(\omega t)$$

Expanding:

$$B\omega\left[\cos(\omega t)\cos(\phi) + \sin(\omega t)\sin(\phi)\right] = -\frac{1}{\tau}\left[A + B\left[\sin(\omega t)\cos(\phi) - \cos(\omega t)\sin(\phi)\right]\right] + a + b\sin(\omega t)$$

$$B\omega\cos(\phi)\cos(\omega t) + B\omega\sin(\phi)\sin(\omega t) = -\frac{1}{\tau}A - \frac{1}{\tau}B\cos(\phi)\sin(\omega t) + \frac{1}{\tau}B\sin(\phi)\cos(\omega t) + a + b\sin(\omega t)$$

$$\left[B\omega\cos(\phi) - \frac{1}{\tau}B\sin(\phi)\right]\cos(\omega t) + \left[B\omega\sin(\phi) + \frac{1}{\tau}B\cos(\phi) - b\right]\sin(\omega t) + \left[\frac{1}{\tau}A - a\right] = 0$$

For this to be true at all times t requires:

$$B\omega\cos(\phi) - \frac{1}{\tau}B\sin(\phi) = 0 \qquad B\omega\sin(\phi) + \frac{1}{\tau}B\cos(\phi) - b = 0 \qquad \frac{1}{\tau}A - a = 0$$

From the first equation we have:

$$\frac{\sin(\phi)}{\cos(\phi)} = \tan(\phi) = \omega\tau$$

Squaring the first two equations and adding the resulting equations yields:

$$B^2\omega^2\cos^2(\phi) + \frac{1}{\tau^2}B^2\sin^2(\phi) - \frac{2}{\tau}B^2\omega\sin(\phi)\cos(\phi) = 0$$

$$B^2\omega^2\sin^2(\phi) + \frac{1}{\tau^2}B^2\cos^2(\phi) + \frac{2}{\tau}B^2\omega\cos(\phi)\sin(\phi) = b^2$$

$$\text{add}: \quad B^2\omega^2\cos^2(\phi) - \frac{1}{\tau^2}B^2\sin^2(\phi) + B^2\omega^2\sin^2(\phi) + \frac{1}{\tau^2}B^2\cos^2(\phi) = b^2$$

$$\left[B^2\omega^2 + \frac{1}{\tau^2}B^2\right]\cos^2(\phi) + \left[B^2\omega^2 + \frac{1}{\tau^2}B^2\right]\sin^2(\phi) = b^2$$

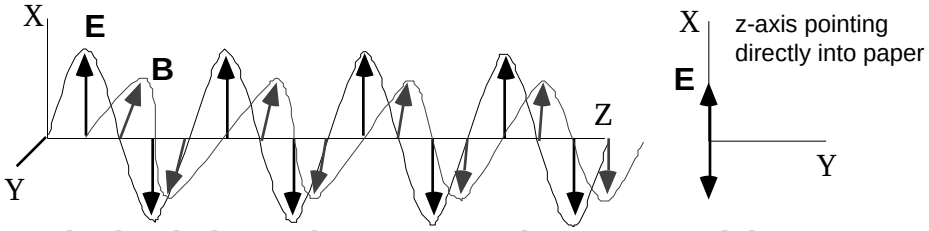
$$\omega^2 + \frac{1}{\tau^2} = \left(\frac{b}{B}\right)^2 \qquad \text{but } \tau = \frac{A}{a}$$

$$m = \frac{Ba}{bA} = \frac{1}{\tau}\left(\omega^2 + \frac{1}{\tau^2}\right)^{-1/2} = \left(\omega^2\tau^2 + 1\right)^{-1/2}$$

Circular Dichroism and Optical Rotation (CS 8.1, vH 10)

Return to a look at *absorption* processes.

Polarization of light



We saw previously that light can be represented as a sinusoidal wave representing its electric field component. In linearly polarized light, all of the electric field component is oriented in a single direction (z above), with the electric field vector oriented uniquely in one direction (x above).

Linear dichroism

We also saw that transitions can be "allowed" along one direction of the molecule and "disallowed" (forbidden) along another. For example, only one orientation of the electric field can induce $\pi \rightarrow \pi^*$ transitions in formaldehyde.

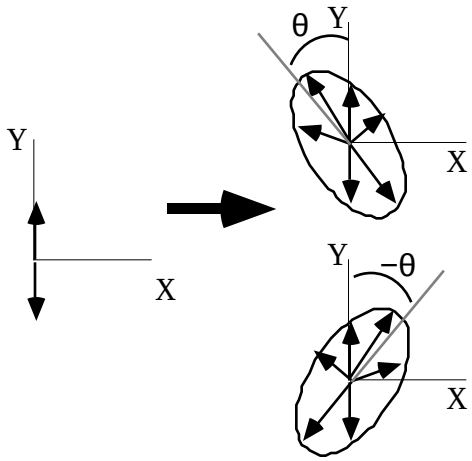
We also saw that for such a molecule in solution, excitation with polarized light will selectively excite only those molecules oriented which are properly oriented. If fluorescence occurs before rotational reorientation, then the emitted light will also be polarized.

More simply, if we look at absorption (*not* fluorescence) in an oriented sample, in particular a sample oriented along one axis, but not along the other two, we can define the dichroic ratio

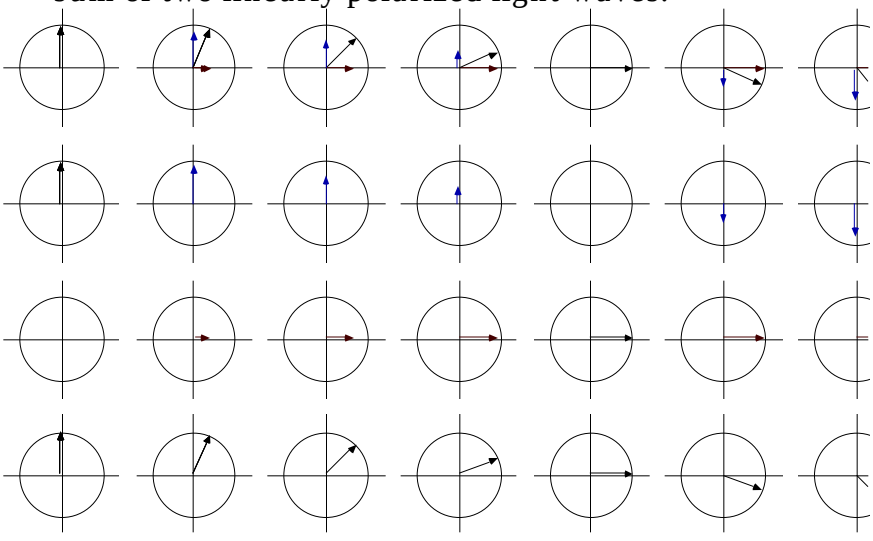
$$d = \frac{A_{||} - A}{A_{||} + A}$$

Example: In DNA, the bases are all oriented with their planes parallel to one another, but rotated to varying degrees within the plane. Since the transition dipole lies in the plane of the bases, only light polarized with its electric field component in that plane will result in an allowed transition, so that $A_{||} = 0$ and $A > 0$. Therefore $d < 0$ and we say that DNA has negative dichroism.

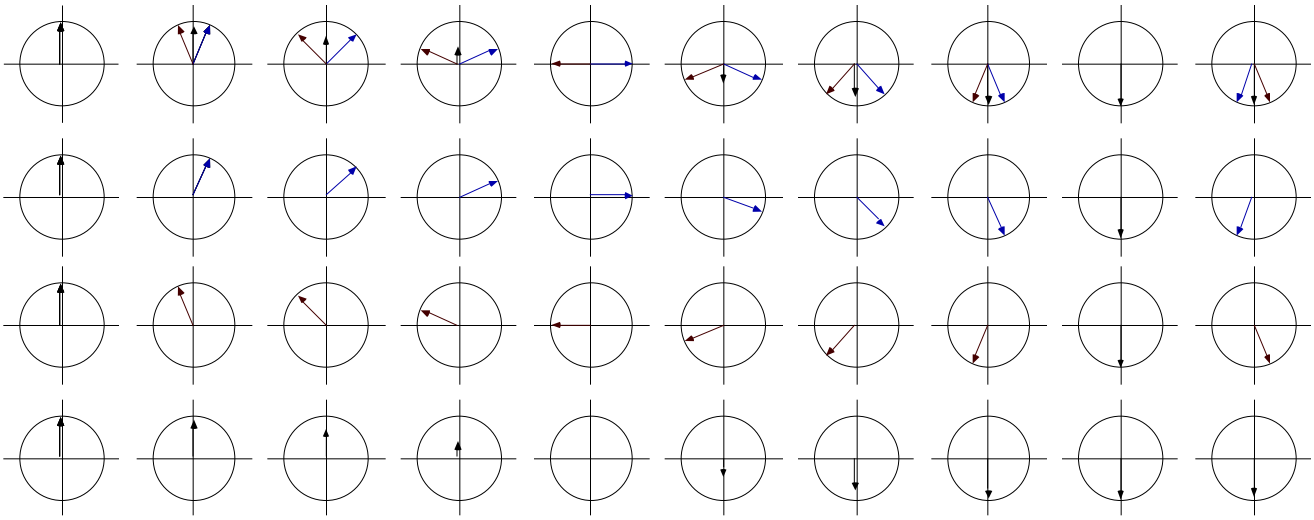
Circular Polarization of light



Circularly polarized light can be represented as the sum of two linearly polarized light waves.



Plane polarized light can be represented as the sum of two circularly polarized light waves.



Absorption phenomenon

Circular Dichroism $\Delta\varepsilon_\lambda = \varepsilon_L - \varepsilon_R$

An optically active molecule can preferentially absorb left or right circularly polarized light. For the moment, accept the fact. We define the **circular dichroism** of a molecule as the difference in the extinction coefficients for left and right circularly polarized light.

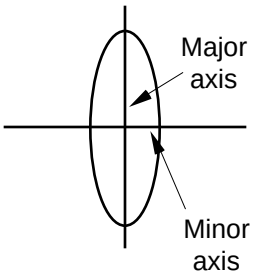
For a given absorption band, this property can very across the band. Such that we can define the overall rotational strength of the molecule as the integrated extinction coefficient difference across the entire band.

$$R \quad \int \frac{\Delta\varepsilon}{\lambda}\, d\lambda$$

Note that CD requires *absorption of light*. But the CD can be positive or negative.

Readily measured. Alternately measure absorption of pure left versus pure right circularly polarized light. Compare numerically.

Ellipticity: $\theta = \arctan \dfrac{\text{minor axis}}{\text{major axis}}$



Starting with linearly polarized lamp incident on a sample. Remembering that linear light can be viewed as the sum of equal parts right and left circularly polarized light, we can predict what will happen when the right circularly polarized component is absorbed more than the left circularly polarized light. The result is elliptically polarized light. The degree of ellipticity can be presented as the ration of the minor axis to the major axis (0, for no difference in absorption for left and right circularly polarized; 1, for complete absorption of one of the components).

Equivalent to circular dichroism: $\theta = 2.303(A_L - A_R)\dfrac{180}{4\pi}$

Refraction phenomenon

Refractive index: $n_r = \dfrac{c}{v} = \dfrac{\text{speed of light in a vacuum}}{\text{speed of light in a medium}}$

This depends on the strength of the interaction between the electromagnetic field of light and the molecules in the medium.

Such interactions depends on the *polarizability* of the molecule.

The refractive index varies strongly with frequency near the frequency of an absorption transition, because the EM field and the light have similar energies.

Circular Birefringence $n_L - n_R$

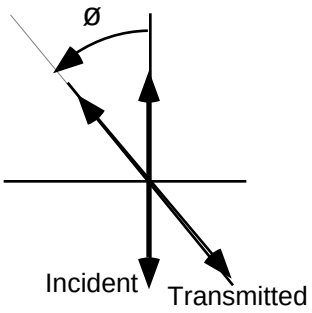
Due to symmetry (or lack thereof) in a molecule, the scattering interaction with it may be different for light polarized circularly either right or left (see below for some insight as to why). As we saw

before, one can measure this difference in scattering by separately measuring the light scattered for either case or by measuring the effect of the sample on plane polarized light.

Circular birefringence is the difference in index of refraction for left and right circularly polarized light. The refractive index difference $n_L - n_R$ results in a difference in phase between the left and right circularly polarized light.

Optical Rotation: ø

Optical rotation is defined as the angle that the major axis makes with the y-axis. That is, it is a rotation of the plane of polarization. Optical rotation as a function of wavelength is called optical rotatory dispersion (**ORD**).



Equivalent to circular birefringence. $ø = \frac{180\ l}{(n_L - n_R)\ \lambda}$

Readily measured. Proteins and DNA at ≈0.1 mM typically rotate the plane of polarization by 0.01-0.1° Current instruments can measure Δø = 0.0001°

Summary

Absorption	Refraction
CD: $\Delta\varepsilon_\lambda = \varepsilon_L - \varepsilon_R$	Circular birefringence: $n_L - n_R$
Intensity (E_o) altered differentially	Phase (β) altered differentially
Ellipticity: $\theta = \arctan\frac{\text{minor}}{\text{major}}$	Optical Rotation: ø

Molecular basis for optical activity

Introduction:

In absorption spectroscopy, the oscillating electric dipole component of light induced a dipole in the absorbing molecule. Moreover, we required that the electron involved be moved into a distribution resembling the excited state. This lead to the quantum mechanical result that the electron went from one *quantized* state to another. For light energies away from the exact required energy, the electric dipole influenced the electron distribution, but not in the exact way to make the probability of the transition very large.

Since the electric dipole of the light is oscillating, so then is this induced oscillating dipole in the molecule.

Note that for an oscillating electric field propagating through space, there is a magnetic component perpendicular to it. We write these:

$\mathbf{E} = \mathbf{E}_o \cos 2\pi\nu(t-\frac{x}{c})$ $\mathbf{H} = \mathbf{H}_o \cos 2\pi\nu(t-\frac{x}{c})$ where x is the position of the molecule.

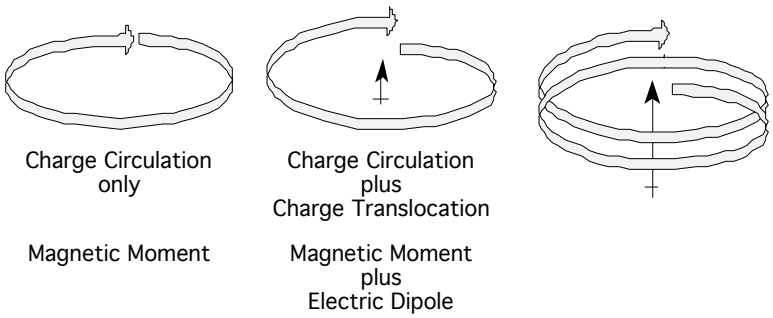
We saw before that the rotational strength in a CD transition is related to the integrated area of the CD spectrum. Quantum mechanically we can describe the rotational strength in a manner similar to the way we described the absorption strength (or probability) in the more simple absorption spectroscopy.

R_{oa} $\langle\psi_b|\underline{\boldsymbol{\mu}}|\psi_a\rangle \bullet \langle\psi_a|\underline{\mathbf{m}}|\psi_b\rangle$ (dipole strength $\langle\psi_b|\underline{\boldsymbol{\mu}}|\psi_a\rangle \bullet \langle\psi_a|\underline{\boldsymbol{\mu}}|\psi_b\rangle$)

where $\underline{\boldsymbol{\mu}}$ = electric dipole operator (of the exciting light)

$\underline{\mathbf{m}}$ = magnetic dipole operator (of the exciting light). This is proportional to the orbital angular momentum of the electron and corresponds to a charge circulation, ie. a "light-induced current loop" analogous to the light-induced electric dipole.

The result of the above dot product is that for a non-zero rotational strength, there must be a light-induced dipole **and** light-induced charge circulation. **Moreover**, the two vectors must have a parallel component (that is, they must not be perpendicular).



The combination of a light-induced dipole with a charge circulation about that dipole is a helical movement of the charge about the common axis. In order to get helical charge movement in a net direction, the molecule must be appropriately **asymmetric**.
Note that the light-induced magnetic dipole is generally much smaller than the light-induced electric dipole, therefore optical activity is typically a **small** effect.

Multi-chromophore interactions

As an example, look at dimer interaction. We have seen this before in the **exciton effect** in absorption spectroscopy. Two induced dipoles in neighboring chromophores can interact via a dipole-dipole interaction.
The rotation strength resulting from the dimer interaction can be expressed as

$$R_{oa} \quad 0.5\ \mathrm{imag}[\ \langle\psi_{1b}|\underline{\boldsymbol{\mu}}_1|\psi_{1a}\rangle \bullet \langle\psi_{1a}|\underline{\mathbf{m}}_1|\psi_{1b}\rangle + \langle\psi_{2b}|\underline{\boldsymbol{\mu}}_2|\psi_{2a}\rangle \bullet \langle\psi_{2a}|\underline{\mathbf{m}}_2|\psi_{2b}\rangle\ ]$$

$$\pm\ 0.5\ \mathrm{imag}[\ \langle\psi_{1b}|\underline{\boldsymbol{\mu}}_1|\psi_{1a}\rangle \bullet \langle\psi_{2a}|\underline{\mathbf{m}}_2|\psi_{2b}\rangle + \langle\psi_{2b}|\underline{\boldsymbol{\mu}}_2|\psi_{2a}\rangle \bullet \langle\psi_{1a}|\underline{\mathbf{m}}_1|\psi_{1b}\rangle\ ]$$

$$\pm \frac{\pi}{2\lambda}\ \underline{\mathbf{R}}_{12} \bullet \langle\psi_{2b}|\underline{\boldsymbol{\mu}}_2|\psi_{2a}\rangle \times \langle\psi_{1b}|\underline{\boldsymbol{\mu}}_1|\psi_{1a}\rangle$$

The important thing to note from the above is that there is a strong distance and orientational dependence on the chromophore-chromophore interaction. Hence CD is very sensitive to the precise geometry of the interacting chromophores.
In fact, this is the basis for most applications of CD. For example, we have seen that the peptide bond is a π system with absorption bands at 220 and 190 nm. For an isolated peptide bond, the extinction coefficient (simple absorption) is very small at 220 nm, but larger at 190 nm.

Secondary structural information

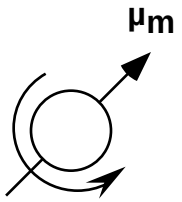
In ordered protein structures such as an α-helix, we have a large number of interacting peptide bonds at characteristic fixed distances and angles. In a β-sheet, the angles and distances are quite different, yet characteristic for that structure. Consequently, the CD spectrum can often be used to extract information regarding protein secondary structure (percent α-helix, β-sheet, random coil, etc.)
For a protein which shows optical activity, the unique combination of circular dichroic contributions is also characteristic of a particular structure for that protein (think of it as a structural "fingerprint"). Even relatively small changes in structure (a conformational change) can have large effects on the CD spectrum. Consequently, CD can be used as a probe of conformational change (in ideal cases, we can sometimes reach conclusions about the disruption of α-helical structure, etc).

Magnetic Resonance Spectroscopies (CS 9; vH 12)

Introduction - Spin

The classical / quantum mechanical spinning top

We discussed previously how a circulating charge produces an electric current. This current in turn produces a magnetic moment $\boldsymbol{\mu_m}$ with an angular momentum, **L**.



This is how most introductory NMR courses introduce *spin angular momentum*. Note, however, that this does **not** refer to an electron moving through space within its orbital - that is called *orbital angular momentum* and will come up later. The classical (not quite correct) explanation for spin angular momentum pictures the electron or nuclear proton "spinning about its own axis).

Note that for an individual particle (electron or atomic nucleus) quantum mechanics dictates that angular momentum is **quantized**. For the electron, angular momentum is quantized in units of the Bohr magneton β_e. Quantum mechanics further dictates that the magnetic moment is related to the angular momentum by a factor g_e.

For electrons: $\boldsymbol{\mu_m} = -\frac{g_e \beta_e}{\hbar}\,\mathbf{L} = \gamma_e\,\mathbf{L}$

where the Bohr magneton $\beta_e = 9.27 \times 10^{-21}$ erg gauss^{-1}

Similarly, for nuclei: $\boldsymbol{\mu_m} = -\frac{g_n \beta_n}{\hbar}\,\mathbf{L} = \gamma_n\,\mathbf{L}$

where the nuclear magneton $\beta_n = 5.05 \times 10^{-24}$ erg gauss^{-1}

Now, the gyromagnetic ratio $\gamma = \frac{\boldsymbol{\mu_m}}{\mathbf{L}} = -\frac{g_e \beta_e}{\hbar}$ (for electrons) or $-\frac{g_n \beta_n}{\hbar}$ (for nuclei)

, and can also be expressed as $\frac{ze}{2m}$ (it is also called the magnetogyric ratio in C&S) .

Quantum mechanics further tells us that **L** is quantized, having allowed values of:

$\hbar[I(I+1)]^{1/2}$ (nuclei)

or

$\hbar[S(S+1)]^{1/2}$ (electrons).

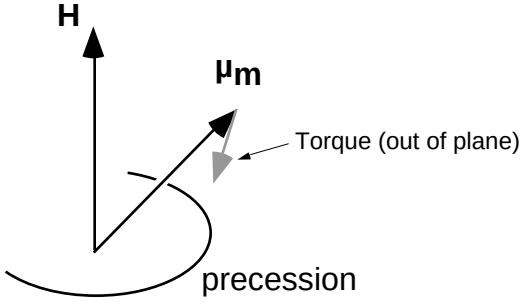
Therefore allowed values of $\boldsymbol{\mu_m}$ are $\gamma\hbar[I(I+1)]^{1/2}$ (nuclei) or $\gamma\hbar[S(S+1)]^{1/2}$ (electrons).

Magnetic moments interact with magnetic fields

The magnetic moment can interact with a magnetic field, **H**, to produce a torque perpendicular to the plane defined by **H** and $\boldsymbol{\mu_m}$.

$\tau = \boldsymbol{\mu_m} \times \mathbf{H}$

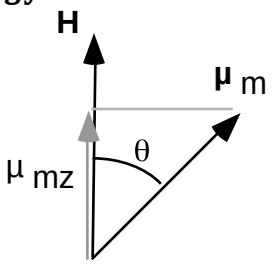
This torque then acts to produce a change (d**L**) in the angular momentum according to



$$\frac{d\mathbf{L}}{dt} = \tau = \boldsymbol{\mu_m} \times \mathbf{H} = \frac{ze}{2m}\,\mathbf{L} \times \mathbf{H} = \mathbf{L} \times \frac{ze}{2m}\,\mathbf{H} = \mathbf{L} \times \omega_o$$

where ω_o is termed the Larmour frequency. We say that the magnetic moment precesses about the applied magnetic field **H** with an angular velocity of precession of ω_o.

Nuclear magnetic moments - allowed values and quantized energy levels



The energy associated with a magnetic moment in an applied field is

$E = -\boldsymbol{\mu_m} \cdot \mathbf{H}$

Note that the dot product really means "the component along..." as shown in the diagram at right.

So that $E = -\mu_m H \cos\theta = -\mu_{mz} H$

μ_{mz} is the component of $\boldsymbol{\mu_m}$ along **H.**

But quantum mechanics dictates that μ_{mz} takes on discrete values according to

$\mu_{mz} = m_I \gamma\!/h$ where m_I = I, I-1, I-2, ..., I-2I

$E = -m_I \gamma\!/h H = -m_I g_N \beta_N H$

Note that the quantum mechanical restrictions on μ_{mz} require that $\boldsymbol{\mu_m}$ is always slightly off axis. Therefore, $\boldsymbol{\mu_m}$ always precesses about $\mathbf{H}_z$, as we saw before.

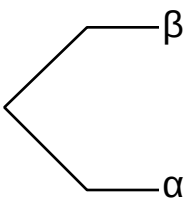
Magnetic properties of selected nuclei

Nucleus	I	γ (rad $G^{-1}s^{-1}$)	% Nat. Abun	Rel. Sens.
^{1}H	1/2	26753	99.98	1.000
^{2}H	1	4107	0.016	0.0096
^{12}C	0			
^{13}C	1/2	6728	1.11	0.016
^{14}N	1	1934	99.64	0.0010
^{15}N	1/2	-2711	0.37	0.0010
^{16}O	0			
^{17}O	5/2	-3627	0.037	0.029
^{19}F	1/2	25179	100	0.834
^{23}Na	3/2	7076	100	0.093
^{31}P	1/2	10840	100	0.066
^{35}Cl	3/2	2621	75.53	0.0047
^{37}Cl	3/2	2182	24.47	0.0027

Nuclei with no net spin (I=0) are useless to us in NMR for obvious reasons. Nuclei with I>1/2, have an additional interaction known as the the nuclear quadrupole interaction, which greatly increases their relaxation rates, and therefore, as we will see later, their NMR linewidths. For this reason, NMR is most simple for nuclei with I=1/2. From the above, we see that the most useful biological nuclei are ^{1}H, ^{13}C, and ^{31}P, with ^{15}N being significantly less sensitive. Other nuclei have proven useful in special cases, notably ^{2}H, ^{19}F, ^{23}Na, ^{35}Cl, and ^{37}Cl.

The relative sensitivity above refers to the expected signal strength for samples with the same number of nuclei and is related to γ (see CS p. 489).

Spin-Spin Interactions - J Coupling



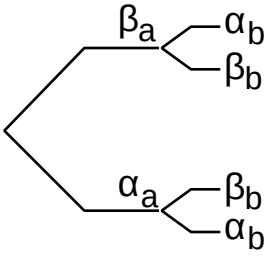
Remember the Nuclear Zeeman interaction:

$$E_{Zeeman} = -m_I \gamma \hbar H = -m_I g_N \beta_N H$$

A spin with m_I = +1/2 is said to be α, while a spin with m_I = -1/2 is said to be β.

m_I = +1/2 (α) refers to a spin aligned *with* the field. This is a favorable interaction. Energy is lowered by this favorable interaction. Conversely for spin with m_I = -1/2 (β).

For the interaction between two non-equivalent spins A and B



$E_{AB} = -m_{I_A} m_{I_B} J_{AB}$ J_{AB} = spin-spin coupling constant

If one nuclear spin is aligned with the field, then the total field that the second nucleus feels is larger. If the second nuclear spin aligns with this field, it is stabilized more than it when aligned with the external field only. In other words, if the spins are both α (or both β), then the energy of the system decreases. However, if the spins are aligned opposite to each other, the energy of the system is increased relative to the energy for no interaction.

This yields the following for the total energy of the system

$E_{Zeeman} = -m_{I_A} g_{N_A} \beta_N H - m_{I_B} g_{N_B} \beta_N H - m_{I_A} m_{I_B} J_{AB}$

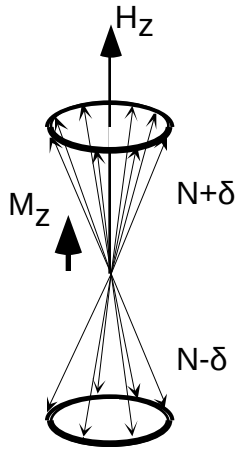
Magnetization

Many spins - bulk magnetization and the NMR experiment

N.B. The following assumes a spin system with I (or S) = 1/2. In this system, there are only two quantum mechanically allowed states (or energies). Analogous arguments hold for systems with I > 1/2, but there will be more allowed states.

Consider a collection of nuclei, each with magnetic moment $\boldsymbol{\mu_m}$. In the absence of a magnetic field, the magnetic moments are randomly oriented.

Now apply a field along the (laboratory) z-axis. The nuclei will tend to align along the axis of the field and will precess about this axis at the Larmour frequency, ω_o. They will eventually populate the allowed quantized energy levels according to the Boltzmann distribution. But how do they get there?



As with absorption energy levels seen before, the spins interchange between energy levels via thermal energy, that is, by interaction with the environment. As a consequence of these random interactions with the environment, the **total** magnetization (**bulk magnetization**) will increase with time according to

$M_z = \bar{M}_z \left(1 - e^{-t/T_1} \right)$ $M_x = M_y = 0$ (the field remains randomly oriented in the xy directions)

where T_1 is termed the "*longitudinal* relaxation time" (longitudinal refers to the direction of the field axis). Again, as we saw in absorption spectroscopy, the non-radiative mechanisms which couple the two states (and determine T_1) can be quite complicated and depend on exactly how the molecule interacts with its environment.

Note that the nuclei are precessing about the field axis, but since they have no phase relationship, the net magnetization in plane remains 0 (all contributions cancel each other in a randomly precessing group of spins).

We can measure T_1 by applying a magnetic field at a given instant in time and then watching the growth of the magnetization along the axis of the field. This is not, however, how T_1 is normally measured.

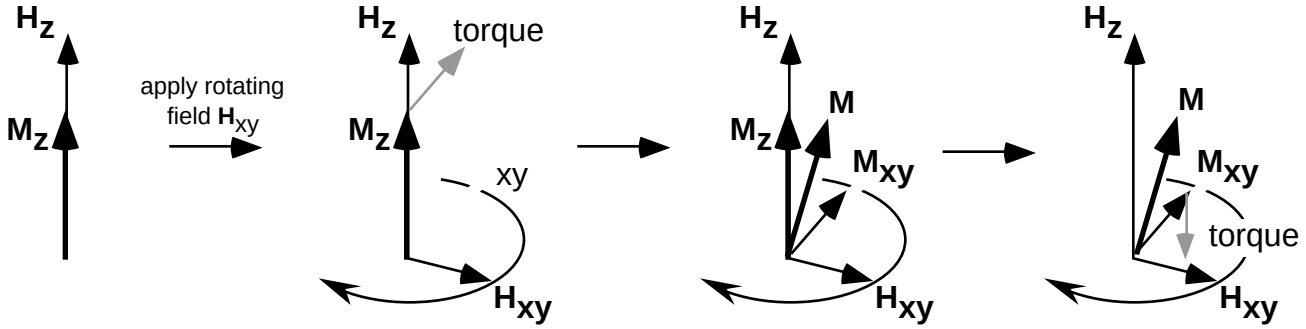
The picture so far presented forms the basis for simple "continuous wave" (CW) NMR spectroscopy. For a system with I (or S) = 1/2, we have a system of two different quantized energy levels (spin up and spin down).

$\Delta E = \gamma \hbar H = \hbar \omega_o = h \nu_o$ for I (or S) = 1/2

Application of electromagnetic radiation of the appropriate energy (now in the radiofrequency region of the spectrum) can induce transitions between levels. Since there is a net population difference, we will have a net absorption of radiofrequency energy, which is measured in a manner analogous to before (now a coiled wire, an antenna, forms the basis for the generation and detection of the "light" (radiofrequency).

Also note that in the absence of an applied magnetic field, there is no difference in energy between the two states (in fact, there is no direction about which to quantize the states). The energy gap between the two states is proportional to the applied magnetic field.

Transverse Magnetization - towards Fourier Transform NMR



In the previous picture, a system of spins under the influence of an applied magnetic field, H_z, gives rise to a bulk magnetization $\mathbf{M}_z$ aligned along the axis of the field $\mathbf{H}_z$. If we were now to apply a second field $\mathbf{H}_{xy}$, perpendicular to the first and rotating at the Larmour frequency, ω_o, there will be a torque exerted on the bulk magnetization vector according to

$\tau = \mathbf{M}_z \times \mathbf{H}_{xy}$

The direction of the torque will be perpendicular to the two vectors, and will tend to tip the magnetization away from the z-axis. This will, in turn, produce a net magnetization perpendicular to the z-axis, $\mathbf{M}_{xy}$. Moreover, this magnetization will be perpendicular to the field $\mathbf{H}_{xy}$. Since the in-plane field is rotating, we say that $\mathbf{M}_{xy}$ and $\mathbf{H}_{xy}$ are 90° out of phase.

But now another torque comes into play. $\mathbf{M}_{xy}$ and $\mathbf{H}_{XY}$ interact to produce a torque perpendicular to their plane (ie., along the negative z-axis). This torque acts to drive $\mathbf{M}$ along the negative z-axis. You can see that this is the mechanism whereby an applied RF field (with its oscillating magnetic field component) can induce transitions between the two quantum mechanical states (aligned with and against the applied magnetic field).

So we see that the application of a field $\mathbf{H}_{xy}$ perturbs the Boltzmann distribution of states quantized along $\mathbf{H}_z$. If we now suddenly turn off the field $\mathbf{H}_{xy}$, the system will return to its equilibrium distribution and by monitoring the return of the bulk magnetization, we can measure T_1 as before (this is a more common approach).

Note also that after the application of the field $\mathbf{H}_{xy}$, there remains a steady state component of magnetization in the xy plane, $\mathbf{M}_{xy}$. After $\mathbf{H}_{xy}$ is turned off, this magnetization component will also decay, but now with a different time course, T_2. With time

$\mathbf{M}_{xy} = (\mathbf{M}_{xy})_0 \, e^{-t/T_2}$

The parameter T_2 is called the *transverse* relaxation time. The same interactions with the environment which give rise to T_1 relaxation contribute to T_2, but additional mechanisms exist for T_2 relaxation. Consequently, $T_2 < T_1$.

In order to maintain the 90° phase relationship between $\mathbf{H}_{xy}$ and $\mathbf{M}_{xy}$, the field $\mathbf{H}_{xy}$ must oscillate at the Larmour frequency ω_o. The energy of the radiofrequency radiation producing $\mathbf{H}_{xy}$ must be

$E = /h \, \omega_o$ which is the energy gap between the levels (see above).

Thus the "light" (RF) producing the transverse field $\mathbf{H}_{xy}$ must oscillate at the frequency ω_o which corresponds to the energy gap between the quantized states.

Steady-state absorption at resonance

We have mentioned above that the application of a field $\mathbf{H}_{xy}$ rotating at the Larmour frequency induces a component of the bulk magnetization in the xy plane. We have also discussed how this field can induce transitions among allowed $\mathbf{M}_z$ states, as for absorption spectroscopy. But as before, there are also thermal processes (non-radiative) which tend to drive the population of states back to the equilibrium Boltzmann distribution. These are T_1 processes.

Similarly, the equilibrium situation in the xy plane is $\mathbf{M}_{xy} = 0$. But the application of $\mathbf{H}_{xy}$ rotating at ω_o causes a net magnetization $\mathbf{M}_{xy}$ rotating 90° out of phase with $\mathbf{H}_{xy}$. Again, thermal processes (T_2) tend to drive this back to the equilibrium value of $M_{xy} = 0$.

As for absorption processes in the optical regime, as long as thermal processes can redistribute the system to near Boltzmann levels faster than you can excite them, you will continue to have a net absorption of energy (the energy is eventually being funnelled off into the thermal processes - heat).

Transverse phase

We instituted the requirement that $\mathbf{H}_{xy}$ be oscillating very near the Larmour frequency ω_o. We see from above $/h\omega_o$ ($h\nu$) corresponds to the energy between states. That makes perfect sense from what we've seen before.

Another way to look at this requirement is to examine the *phase* relationship between the involved vectors. When $\mathbf{H}_{xy}$ is applied, it generates a torque on $\mathbf{M}_z$ to create $\mathbf{M}_{xy}$ in plane and at 90° to $\mathbf{H}_{xy}$. $\mathbf{M}_{xy}$ will continue to precess at ω_o. As long as $\mathbf{H}_{xy}$ also rotates at ω_o, the two will remain at 90°. If $\mathbf{H}_{xy}$ is rotating at $\omega \neq \omega_o$, then the angle between them will slowly drift from 90°. But $\mathbf{H}_{xy}$ will continue to exert a torque on $\mathbf{M}_z$ and so will create a new $\mathbf{M}_{xy}$ at 90° to $\mathbf{H}_{xy}$. But this new $\mathbf{M}_{xy}$ will of course not align with the previously produced $\mathbf{M}_{xy}$ (no longer at 90° to $\mathbf{H}_{xy}$). You can see that the magnetization generated in the xy-plane will be random and will cancel itself out. The *net* magnetization $\mathbf{M}_{xy}$ will be 0.

The Bloch Equations Condensed

Cantor & Schimmel discuss an approach to understanding NMR first proposed by Bloch. We will skip over this formalism although students interested in seriously applying NMR will do well to read this chapter carefully (p. 493-498).

From this approach we have a formula for NMR signal strength:

$$\text{Signal} \quad N \frac{\mu_{mz}^2 H_z^2}{kT} \frac{\gamma^2 H_{xy} T_2}{1 + T_2^2(\omega_o - \omega)^2 + \gamma^2 H_{xy}^2 T_1 T_2} \qquad I=1/2 \text{ only}$$

Note that, as expected, the signal strength is directly proportional to the number of spins in the sample, *i.e.* to the sample concentration. Also note that the signal increases as H_z^2, such that a doubling of field strength quadruples the signal.

Brief aside: "rates" (k) vs. "lifetimes" or "half-lives" (T_1 and T_2)

It is important to note that lifetimes are inversely proportional to rate constants $k = \frac{1}{T}$

Thus, <u>faster (larger) relaxation rate</u> = <u>shorter (smaller) relaxation time</u> (and vice versa).

T_1 - Longitudinal Relaxation - a.k.a. <u>Spin-Lattice Relaxation Time</u>

What happens when T_1 is "Large" ("*long*")? From the above equation: signal $1/T_1$

When the longitudinal relaxation time is long, thermal processes do not reequilibrate the levels efficiently so that application of $\mathbf{H}_{xy}$ causes the two levels to be more equally populated and we have fewer **net** transitions in an absorption direction. Signal <u>decreases</u>. This effect is called <u>*saturation*</u> and will prove very important in the future.

What is the basis of T_1 relaxation?

We know that non-radiative processes are due to interactions with the environment. For optical absorption, the mechanism of non-radiative mixing of the ground and excited states is often the interaction of the molecule with fluctuating dipoles in the environment. In the case of T_1, the mechanism of mixing is via interaction with fluctuating (randomly oriented) magnetic fields in the medium. To the extent that a neighboring field has a magnetic component along $\mathbf{M}_{xy}$ which is oscillating at the Larmour frequency ω_0, it can act just like $\mathbf{H}_{xy}$ to induce a transition between the quantized m_z states.

We call the environment the "**lattice**" (hence "spin-lattice relaxation) and its nature strongly effects T_1. In liquids or gases there is substantial molecular motion such that the local magnetic fields produced by neighbor molecules have a wide frequency distribution. This means that there will be a number of oscillating fields at ω_0 and T_1 can therefore be small ("fast"), typically <10 sec. In a solid, those motions are severely restricted so that the low frequency fluctuations can be $<< \omega_0$. In this case T_1 can be hours.

N.B. - the rotation of neighboring molecules giving rise to fluctuating magnetic fields is *relative*, that is, it doesn't matter which of the two molecules is doing the rotating. Thus, if we are measuring T_1 of methyl group protons, even though the environmental magnetic fields may be rotating slowly with respect to the molecule (protein) to which the methyl group is attached, the methyl group itself may be rotating quite readily. Thus from its point of view, the environment is rotating rapidly - the magnetic fields that it "sees" are fluctuating at a high rate. Thus different atoms within the same molecule may have very different relaxation rates.

T_2 - Transverse Relaxation - a.k.a. Spin-Spin Relaxation Time

The frequency ω_0 with which spins precess about the applied field is a function of that field $\mathbf{H}_z$. But the effective field that an individual spin "sees" is influenced by the environment, and so can be written $\mathbf{H}_z + \Delta\mathbf{H}_{loc}$. Just as the field will be heterogeneous due to local environement, so too will be ω_0. This means that spins in the xy-plane will precess at individual frequencies, $\omega_0 \pm \omega_{loc}$.

In addition, this group of spins will not retain their phase relationship and M_{xy} will decay once the tipping field is turned off.

This loss of phase occurs via two mechanisms:

1) $\Delta\mathbf{H}_{loc}$ due to small time-dependent fluctuating fields from the local environment. In other words, immediately after the spin "packet" is tipped into the xy plane, the spins all have the same phase. But with time, one spin may have its $\Delta\mathbf{H}_{loc}$ altered, changing at the same time, its phase. Even if it returns to its original $\Delta\mathbf{H}_{loc}$, its phase "memory" is lost.

2) spin exchange with neighboring nuclei. In this latter case, an two neighboring nuclei of opposite spin can exchange the sign of their spins (analogous to energy transfer we've seen before). But in the process of this exchange, the phase relationship that each spin had is lost. Similarly T_1 transitions between m_z levels also cause a change in spin and contribute to T_2 dephasing - T_2 will therefore always be at least as fast as T_1.

NMR Linewidths

Returning to the equation for signal strength, we can simplify it somewhat to reveal the effects of T_1 and T_2 on the NMR signal.

$$\text{Signal} \quad \frac{T_2}{1 + T_2^2(\omega_0-\omega)^2 + \gamma^2 H_{xy}^2 T_1 T_2}$$

$$\text{Signal}_{max} \quad \frac{T_2}{1 + \gamma^2 H_{xy}^2 T_1 T_2}$$ maximum signal at resonance, $\omega = \omega_0$ (the "peak" of the signal)

We can combine the two equations above to determine the linewidth $\Delta\omega = \omega_o - \omega$ at which the signal is half of its maximum value.

$$\Delta\omega_{1/2} = \frac{2}{T_2}(1 + \gamma^2 H_{xy}^2 T_1 T_2)^{1/2}$$

From the above, we can see that:

For constant T_1, the linewidth *increases* (signal *broadens*) for decreasing values of T_2.

For constant T_2, the linewidth *decreases* (signal *narrows*) for decreasing values of T_1. Also note that the linewidth is independent of H_z.

Finally, note that when $\gamma^2 H_{xy}^2 T_1 T_2 << 1$, $\Delta\omega_{1/2} = \frac{2}{T_2}$ and Signal_{max} T_2

Molecular Rotation

If a molecule (or a molecular substituent) is rotating quickly enough in space, then during the timecourse of these measurements, a nuclear spin will only "see" an average environment (just like the rotational averaging we saw for Förster energy transfer). In this case, $\Delta\mathbf{H}_{loc}$ is the same for all nuclei of that type and T_2 dephasing is much less. Similarly neighboring nuclei spend too little time near each other to exchange spin. In this *extreme narrowing limit*

$1/T_2 \approx 1/(2\ T_1)$

A factor which often limits NMR of proteins is in fact the slow rate at which the macromolecule (and therefore the fixed nuclei within it) tumble randomly in solution. Again, note that even for a very slowly tumbling protein, protons on a surface methyl group may rotate at a much higher rate (and show sharp resonances). In solids we have the extreme limit of limited rotation, and consequently we generally see very broad spectra for solids. However, it is now possible to mechanically spin the sample at a frequency greater than T_2^{-1}.

NMR - Properties of Molecules

Chemical Shift

So far, we have implicitely dealt with groups of identical nuclei. They may have been *randomly* in slightly different environments (thus giving rise to relaxational effects), but on average they all felt the same field. But in a real molecule with multiple nuclei, we know that some nuclei are different environments than others *in a well-defined way.* For example, methyl protons are in a different environment than are amide protons. And in a given molecule or protein, certain amide protons will be in different environments than are other amide protons (interacting with solvent in a random coil, H-bonded to a amide carbonyl in an α-helix, etc.).

For this reason, not all protons feel the same net H_z. We can write:

$$H_z' = H_z - H_z\sigma = H_z(1-\sigma) \qquad \sigma = \frac{H_z - H_z'}{H_z}$$

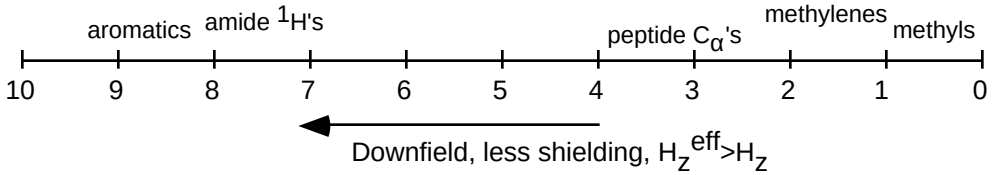
σ describes how much the local environment adds to (or subtracts from) the applied field H_z.

More generally, the additional field produced by the environment is compared to the effective field in a reference standard sample. In this case

$$\delta = \frac{H_{ref} - H_{samp}}{H_{ref}} \times 10^6 = \frac{\nu_{ref} - \nu_{samp}}{\nu_{ref}} \times 10^6$$ expressed in *parts per million.*

where H_{ref} is the effective field felt by the reference nucleus and H_{samp} is that felt by the sample nucleus. Alternatively, one can speak about resonance frequencies, ν, at constant field.

Thus we have an NMR *spectrum*, most often plotted as a function of frequency or ppm. Individual protons in a molecule will resonate at individual frequencies in the spectrum.



At a field strength of ≈100,000 guass, most protons resonate over a range of ≈5,000 Hz, centered around 500 MHz. Values of δ range between 0 and 10 ppm (relative to the protons of sodium 2,2-dimethyl-2-silapentane-5-sulfonate, DSS, or tetramethylsilane, TMS).

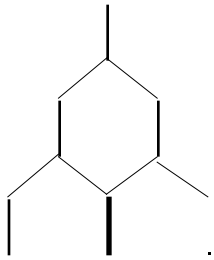
Ring current shifts - aromatic amino acids

We will not go into a detailed analysis of which groups show what type of shifts, but one effect can be very important in the study of proteins. For an aromatic compound such as benzene, we have seen that electrons reside in delocalized, circular π systems. In the presence of an applied magnetic field, these electrons are driven in a circular motion within these orbitals. This circulating charge produces an inductive magnetic moment. The resulting field adds to the applied field *outside* of the aromatic ring, and opposes the applied field within (above and below) the ring. Thus ring protons, which protrude on the outside edge of aromatic rings found in Phe, Tyr, etc, feel a field larger than applied magnetic field. This leads to a shift in δ of as much as 8-10 ppm.

Spin-spin interactions - splitting of resonance lines.

The environmental effects discussed above are due to interactions between nuclear spins and other factors in the environment - the magnetic field produced by an oscillating electric dipole or the magnetic moment produced by circulating electrons. Another very important interaction is that between two nearby spins. The magnetic moment associated with one spin adds to the effect magnetic field felt by the other and vice versa. From the point of view of one nuclear spin, the neighbor spin can be either aligned with or against the applied field (remember that it is quantized). So the field felt by the first spin is $H_z \pm 0.5J$, where J is called the (spin-spin) coupling constant and the *neighbor* spin has I=1/2. In the spectrum, this results in two absorbance lines centered at the original frequency and split by J.

You can readily see that for the interaction of one spin with two *identical* neighboring spins (I=1/2), the single transition will be split into two and each of those again split into two. Since the splittings are identical, we get the familiar 1:2:1 pattern. If the two neighboring spins are not identical, then the resulting pattern will be a doublet of doublets. Try this one for yourself.



Spin-spin splitting is very useful in NMR in that it tells us who is next to whom in the molecule. Such information is absolutely essential in assigning protein spectra. As you will see, the detailed mechanism of this interaction can be either through-space or through-bond.

Spin-spin splitting can sometimes get in our way as well. We have seen before that if a particular contributor to a spin's environment is fluctuating in its properties faster than the time course of the measurement, then the spin "sees" only the average of that fluctuation. In the case of one spin splitting another (different) spin, if we were to apply a second oscillating field at the resonance frequency of the second spin, then the first spin will see only an average - in this case since the two choices are spin up and spin down, the average will be zero. In this way, then the first spin is no longer split by the presence of the second spin. This general phenomenon is called **decoupling**.

Chemical Exchange

We have seen above that if a nucleus is flipping its spin very rapidly, then its neighbors will see an average of the flipping states (no spin, in this case). This averaging is a general effect. In fact, if a nucleus is jumping back and forth between two (or more) environments, the NMR properties of that nucleus will be an average of the two states (specifically, an average *weighted* by the proportion of time spent in each state).

Slow exchange

Consider a nucleus which can be in two chemical environments: A or B. Designate the lifetime in state A as τ_A and the lifetime in state B as τ_B. Also let the resonant frequencies of the nucleus in the two environments be ω_A and ω_B.

For conditions of "slow exchange," $\tau_A >> \frac{1}{\omega_A - \omega_B}$ AND $\tau_B >> \frac{1}{\omega_A - \omega_B}$

two distinct lines are seen in the NMR.

Moreover, their T_2 relaxation times are given by $\frac{1}{T_{2i}} = \frac{1}{T_{2i}^0} + \frac{1}{\tau_i}$

where $\frac{1}{T_{2i}^0}$ is the relaxation time of the nucleus in site i in the absence of exchange.

Fast exchange

If however the lifetime of the nucleus in each state is shorter than the precession time, then the lines will merge into a single line. Under these conditions, the T_2 relaxation time is

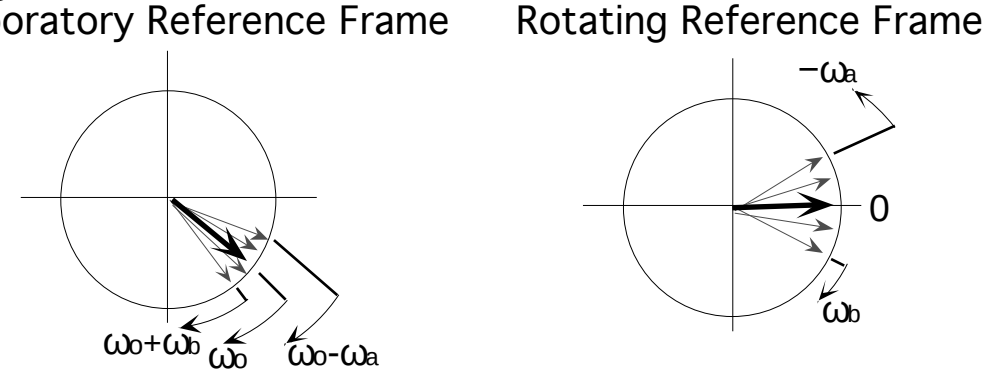
$$\frac{1}{T_2} = \chi_A \frac{1}{T_{2A}^0} + \chi_B \frac{1}{T_{2B}^0} + \chi_A^2 \chi_B^2 (\omega_A - \omega_B)^2 (\tau_A + \tau_B)$$

where χ_A and χ_B are the fractions of the nuclei in states A and B, respectively. This effect is called **exchange broadening**.

This phenomenon can often be used to measure *dynamics* in biological systems.

Fourier Transform NMR

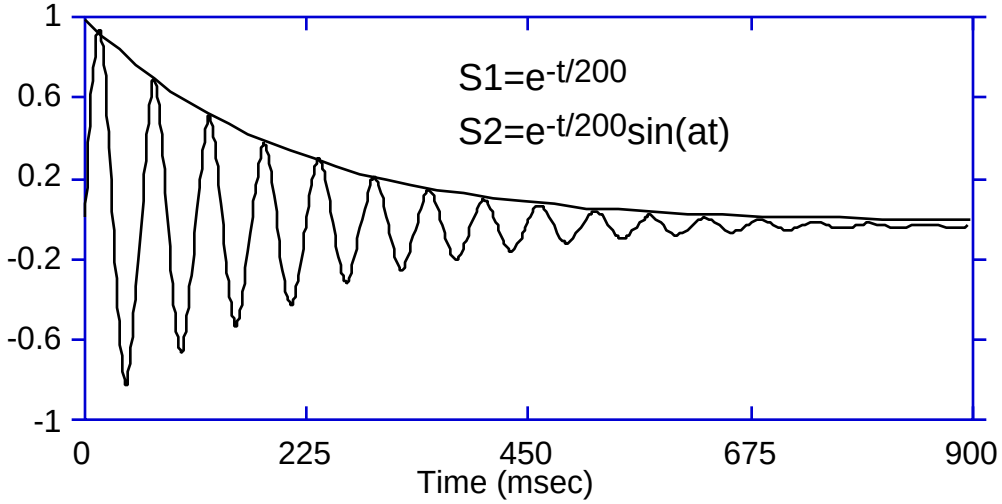
The Rotating Frame



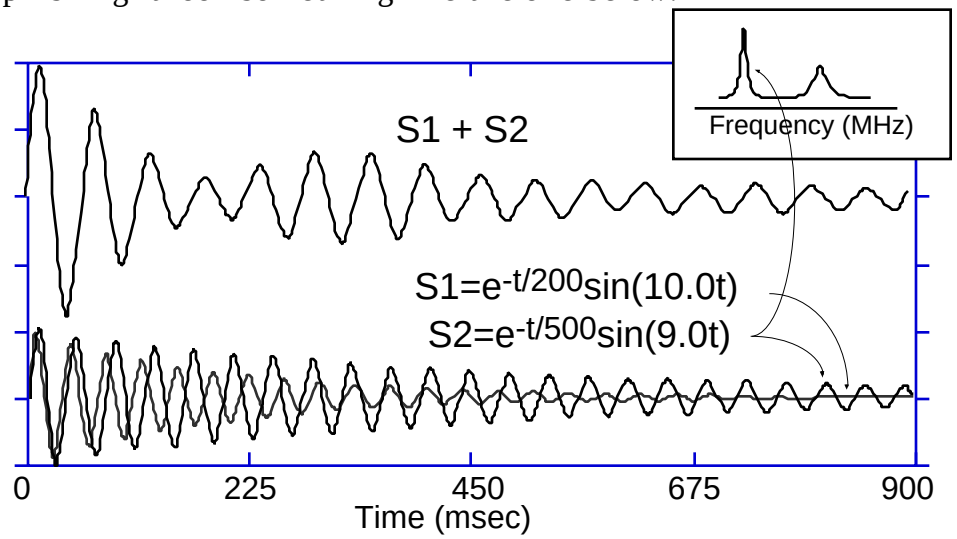
We have now seen that in a real NMR sample, there is a magnetization vector for each nucleus in the sample and that each of these vectors precesses about the applied field at a slightly different rate due to differences in environment. The figure at left illustrates several precessing spins in the xy-plane. If we fix our reference system on the central spin (*i.e.*, rotate our coordinate system at a frequency ω_0, then the rotating spins appear as at right. The spin that was precessing at exactly ω_0 is now stationary. Spins precessing more slowly, now precess in a negative direction and those precessing at a higher frequency precesses in a positive direction. Note immediately following the initial 90° pulse which rotated the magnetization away from H_z, all spins are aligned. With time those that precess faster get away from those that precess more slowly. The actual precession angle as a function of time is given by $\omega_1 t$.

Free Induction Decay

We saw before that if we tip the magnetization into the xy-plane, a component of the magnetization in the xy-plane precesses about the field. If we place a radio receiver antenna ("coil") in the xy-plane, this precessing magnetization will induce a sinusoidal current in the receiver, corresponding to its precession. We also saw that the *net* magnetization will slowy decay, as T_2 processes lead to a gradual dephasing of the spins. The resulting free induction decay, or **FID**, may look something like the figure below.



If there is more than one spin, then the signal will be a combination of more than one sine wave. Each with its own characteristic frequency and relaxational properties. The FID for two spins might look something like the one below.



Via a mathematical transformation of the data known as a Fourier transform, one can decompose the above FID into a frequency domain spectrum in which each peak in the NMR spectrum corresponds to a spin-generated sine wave above, and the linewidth of the signal reflects the relaxational properties we examined earlier (from the Bloch equation result above).

This is the simplest basis for an NMR experiment. But it gets more complicated... And the information gets richer...

First, let's look more at the Fourier transform. Note in the above figure that the sine wave which decays more rapidly (ie. the one with the shorter T_2), gives rise to a more broad signal in the resulting Fourier transform frequency domain spectrum. This is what the Bloch equations told us should happen.

We can manipulate FID's (and often do) in the computer to artificially alter the apparent linewidths of the NMR resonances. We saw before that it is an inherent property of the Fourier transform that fast decay in the time domain gives rise to broad resonances in the frequency domain, and of course the opposite is true. Indeed, if our spectrum is characterized by broad lines and we want to be able to distinguish peaks better, we can artifically multiply our expermental FID to decrease the early time signals and increase the later ones. This is typically done by applying a Gaussian muliplication to the FID. The result is an FID which decays more slowly. When we Fourier transform this, we get a spectrum with sharper peaks!

Why don't we just keep doing this more and more to get infinitely resolved peaks?! Notice that the beginning of the FID has more "information" in terms of the sine wave. The end of the FID has much less (typically we collect an FID until the signal has decayed completely). In the manipulation we did above, we decreased the early (signal-rich) part of the FID and increased the late (noise rich) part of the FID. Consequently, as we do this, we decrease the signal-to-noise ratio of our final spectrum. This limits how much we can artificially increase the resolution. There is no free lunch.

In fact, if we have a spectrum with good resolution but poor signal to noise, we can do the opposite. We can apply a exponential decay weighting function to our FID. This increases the signal-rich part of our FID and decreases the noise-rich part. The signal-to-noise ratio of the final spectrum increases (but our peaks broaden somewhat).

90° / 180° Pulses

90° PULSE - If we apply a very intense field H_{xy} for a very short (but specific) period of time, we can tip essentially all of the magnetization away from M_z. The angle by which the magnetization is tipped is given by $\theta = \gamma H_{xy} \Delta t$ (this can be readily derived from what we learned about torque on magnetic moments above). So we see that the angle is a function of both the intensity of the field and the time for which it is applied. If we tip the magnetization 90° (a 1-200 µsec pulse under typical conditions), after the transverse field is gone, there will be magnetization in the xy-plane rotating at ω_0. As the T_2 phase decays, so too will the net magnetization in the plane (and so then will our signal). This signal is known as the **free induction decay**.

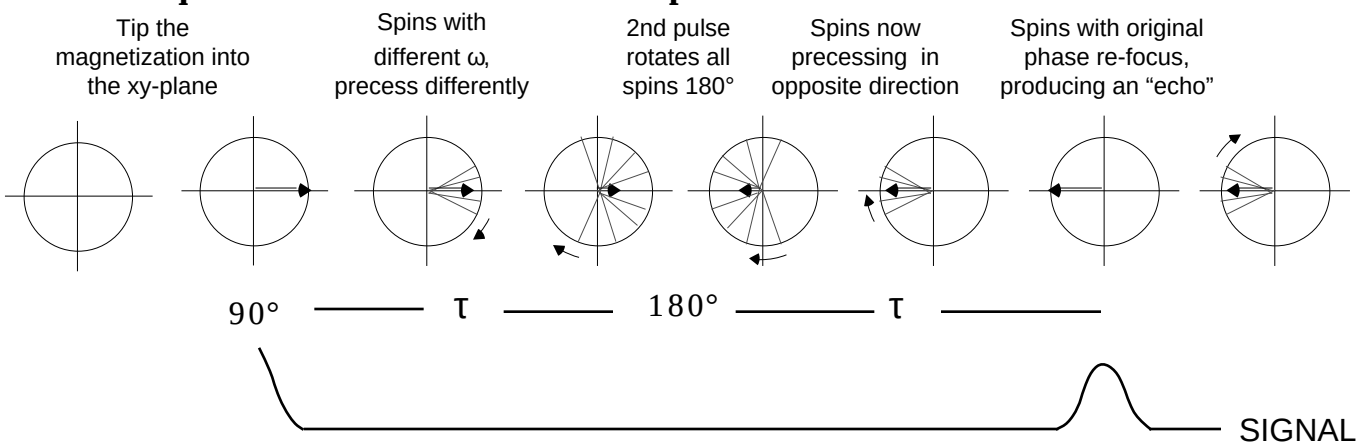
180° PULSE - In a similar fashion, we can apply twice the field strength (or the same field for twice the time) and tip the magnetization all the way around 180°. Note that in this experiment, we have taken the whole system from its Boltzmann distribution producing the original M_z to one with a net $-M_z$. At the end of the pulse, the system will slowly decay back to its Boltzmann value of $+M_z$ according to T_1.

Pulse Sequences: 180° - τ - 90° measures T_1

We can exploit a combination of pulses above, to measure T_1 and T_2. We just say that following the application of a 180° pulse, the net magnetization is changed from $+M_z$ to $-M_z$ and that with time this decays back to $+M_z$ according to T_1. But our detection scheme has relied on detecting a rotating magnetization in the xy-plane. At no time in this sequence, is there net magnetization in the xy-plane.

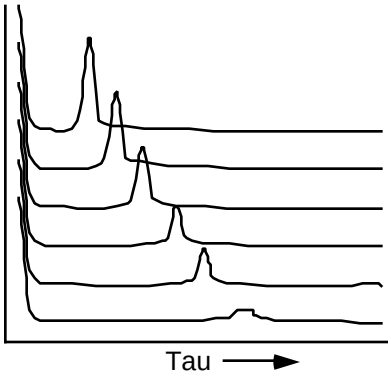
Now look at what happens if we apply a 180° pulse, followed immediately by a 90° pulse. This is essentially the same as a 270° pulse. All of the magnetization will be sent to the xy-plane and we will see a large signal in our detector. If however, we apply a 180° pulse and wait a short period of time (τ), the magnetization $-\mathbf{M}_z$ will decrease according to e^{-t/T_1} . If we then apply a 90° pulse, we will tip the remaining magnetization into the xy-plane and see a signal (now smaller, reflecting the fact that the tipped M_z was smaller). This is called a 180-τ-90 pulse sequence.

Pulse Sequences: 90° - τ - 180° - The "Spin Echo"



The above "pulse sequence" is the basis for much of modern FT NMR. It is very important to examine what is happening during the time course of this pulse sequence. We first apply a 90° pulse to tip the bulk magnetization into the xy-plane. As we have seen, the spins will precess about the field, but each at a slightly different ω due to slight differences in the chemical shift of each spin. If we wait a period of time τ the bulk magnetization will have spread out in the plane, reducing the overall signal, but in the absence of dephasing, each spin is precessing at a different, but *well-defined* frequency ω. If we then apply a 180° pulse, each spin will be rotated about y as shown above. Each spin is still precessing about the applied field at its own frequency, but now in the opposite direction that it had been. These means that each spin will retrace its steps and after a time (exactly) τ, they will all end up together (rotated 180° from where they were placed by the initial 90° pulse). Thus the spins will coalesce, returning the signal to its maximum value and producing an *echo*.

But we know that in a real system, T_2 processes will occur, leading some spins to lose their original phasing. Such spins will not coalesce with the rest to produce the echo at time 2τ. Thus, if we plot the echo intensity as a function of τ, the resultant trace will decay as $e^{-t/2\tau}$ as shown at right.

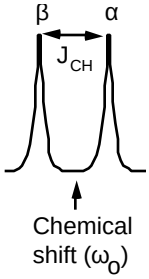


Field Inhomogeneity

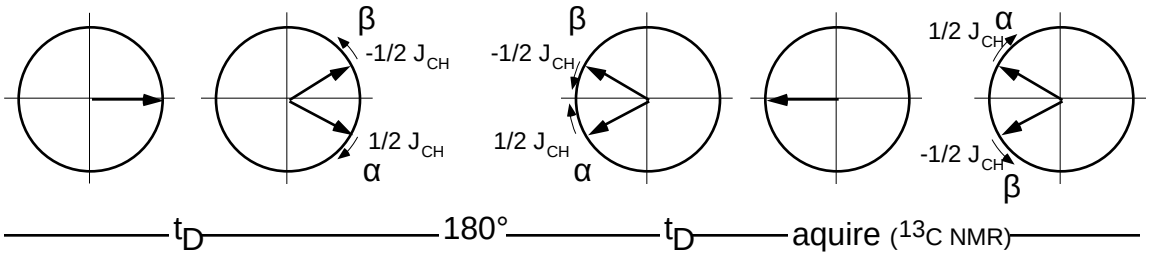
This pulse sequence is useful for another reason. NMR magnets are not perfect and it is impossible to design one such that every spin in the sample sees exactly the same H_z. One part of the tube will have a slightly different H_z (remember it only takes one part in a million to mess things up...). This means that protons which are chemically the same (eg. methyl group protons at the "3" position of our molecule) *should* precess at the same ω_o in fact will have different precession frequencies. This means that they no longer precess together and their bulk magnetization in the xy plane decays more rapidly than it intrinsically should - the signal for that proton is broadened and if we were to measure the simple decay to get T_2 we would get the wrong value.

In the pulse sequence above, after the first 90° pulse, these spins separate from each other in the plane. However, after the 180° pulse, they refocus exactly! Only true dephasing will keep them from refocusing and producing our spin echo. Thus measuring T_2 by the spin echo method gives us the true T_2.

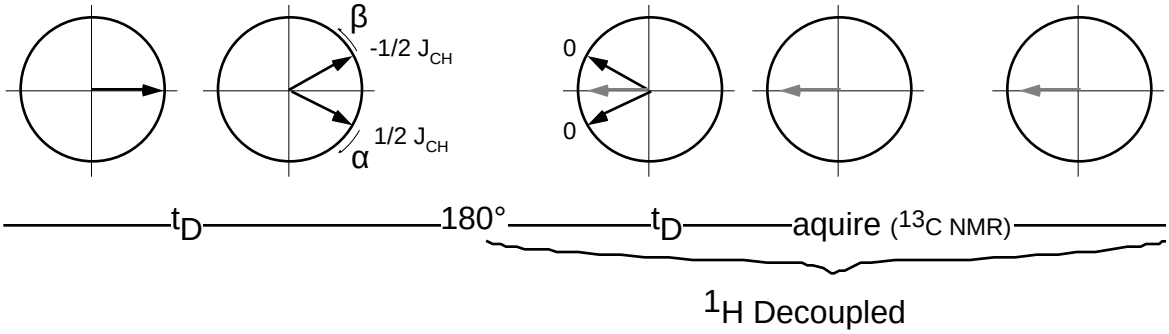
J-Modulated Spin Echo (Heteronuclear)



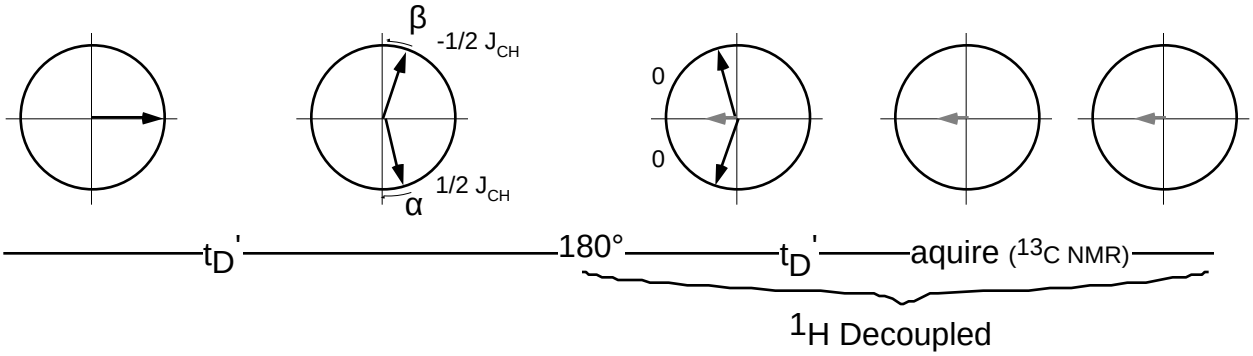
We have discussed how spin-spin interactions occur because one nucleus "feels" the magnetic field produced by a neighboring nucleus. Let's consider not just two different kinds (chemical shifts) of spins, but two different kinds of nucleus (eg. ^1H and ^{13}C) - heteronuclear coupling. As an example, let's look at the ^{13}C NMR spectrum of chloroform: $CHCl_3$. We know that the ^{13}C resonance will be split into two by interaction with the ^1H nucleus (which is either spin up or down). The ^{13}C resonances precess at $\omega_o \pm J_{CH}/2$, or in the rotation frame, they precess at $+J_{CH}/2$ and $-J_{CH}/2$.



But now, let's repeat the experiment but applying ^1H decoupling throught the second delay (t_D) and the aquisition (but *not* during the initial delay. When decoupling is on, the two ^{13}C spins now precess at the original (chemical shift) ω_o (in other words they *do not* precess in our rotating frame). We get an FID from them, corresponding to a single peak centered at the chemical shift. But notice that the intensity of this peak is less than what it would have been had there been decoupling throughout the entire process.



Now let's lenghten the delay time t_D:



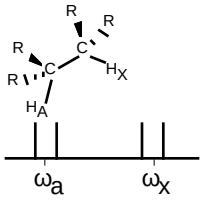
In fact, the extent to which this signal is decreased is a function of the delay time t_D and the J-coupling precession frequency ($J_{CH}/2$). Depending on their relationship, the resulting "static" vector can be large ($1/2\ J\ t_D = 0,2\pi$), zero ($1/2\ J\ t_D = \pi/2,3\pi/2$), or negative ($1/2\ J\ t_D = \pi$).

We have talked before how the FID is simply a collection of sine waves and that when we Fourier transform this "time domain" we get a "frequency domain" spectrum. We can think of t_D as a new time variable. By convention, since t_D comes first it is often

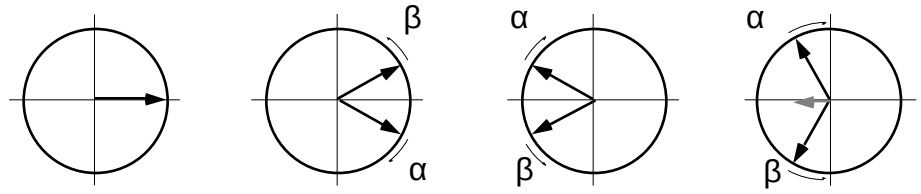
called t_1 (not to be confused with T_1 and the FID time domain is called t_2. When we FT time domain t_2 in this case each resonance will vary sinusoidally with t_1, with the frequency of that oscillation determined by the J/2 coupling (modulated by J coupling). So if we FT the entire set of spectra obtained obtained in the aquistion, we will get a frequency domain in this second dimension. 2 dimensional NMR!
Note that the extent to which the spins have precessed during the delay is a function of their spin-spin (in this case) coupling strength and the delay time. This coupling will be different for different spins with different couplings.

Homonuclear J-Modulated Spin Echo

Now let's look at the same situation, but one in which protons split protons (homonuclear). Consider two spins A and X.

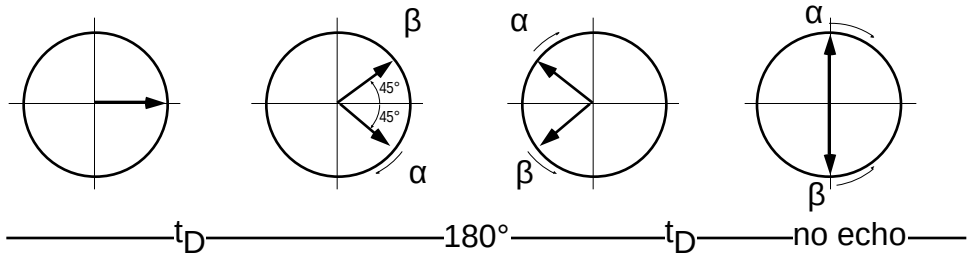


Let's look at our spin echo pulse sequence for the spin H_A. Use the rotating frame ω_a.

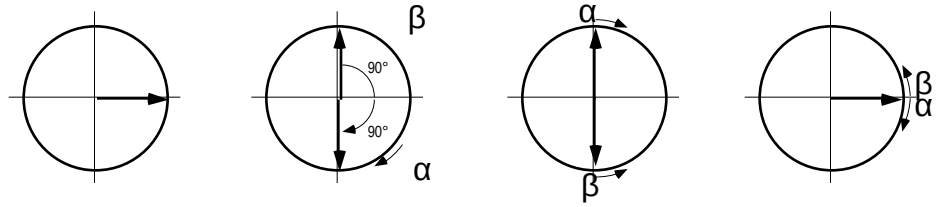


t_D — 180° — t_D — look at echo (^{1}H NMR) or aquire (^{1}H NMR)

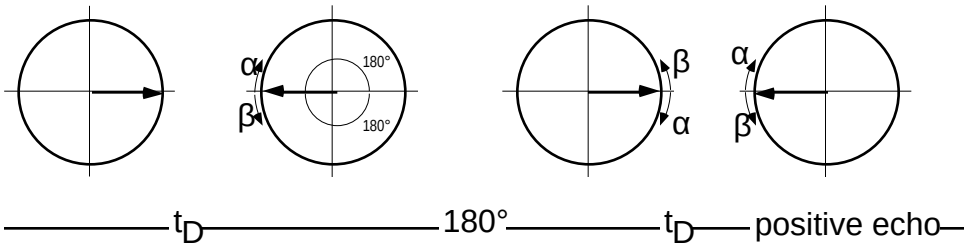
As before, we do a 90° pulse, followed by a delay t_D. During this time, the spins precess in opposite directions as before.
Now apply the 180° pulse. As before, the spins are flipped, but note that the spins that these spins are coupled to are also flipped (in other words the the H_A spin above labeled α originally saw the neighboring X spin as "up" - hence the term α). Now that same spins sees its neighbor "down" and so we now call that H_A spin β. The direction of the subsequent precession depends on this and do the spins precess in the direction opposite to what we saw before. They will in general ***not*** produce an intense echo after a delay t_D. You can also view this by noting that the spins are now **out of phase** with respect to before (by exactly $4\pi\, t_D\, J_{ax}$ radians).
Look at specific delay times:



t_D — 180° — t_D — no echo



t_D — 180° — t_D — positive echo



Concept: Population transfer

Measuring Through-Bond Couplings - COSY

We saw before that spin-spin couplings (J-couplings) measure the interaction between two nuclei. In particular, these interactions occur *through-bond* - they require that the nuclei be connected by a small number of covalent bonds. Therefore, J-coupling information is valuable in assigning the covalent structure of a molecule.

A *two-dimensional* NMR technique called COrrelated SpectroscopY (COSY) measures J-couplings in a single set of pulse experiments. We do not have time to go into the mechanics of how this is done, but the end result is a two-dimensional spectrum in which chemical shift appears on both in-plane axes. The vertical axis contains a "spectrum" in which peaks occur only in the two one-dimensional spectra at at the intersections of two J-coupled transitions.

Measuring Through-Space Couplings - NOESY

We have seen before that two dipoles can interact in a purely *through-space* manner (for example, in Förster energy transfer). A similar interaction occurs in NMR to produce (mainly) relaxational effects. Another two-dimensional approach exploits this effect, such that cross-peaks in the two-dimensional spectrum reflect nuclei coupled via a dipole-dipole mechanism. This effect is called the Nuclear Overhauser effect, the spectrum is called NOESY.

ESR

Electronic Zeeman Interaction

$E_{Zeeman} = +m_s \gamma \hbar H = +m_s g \beta H$

A spin with $m_S = +1/2$ is said to be α, while a spin with $m_S = -1/2$ is said to be β.

$m_S = +1/2$ (α) refers to a spin aligned *with* the field (as in NMR). Since the charge of an electron is negative, all interactions are opposite that which we described previously for interaction with an applied magnetic field. Energy is increased by this unfavorable interaction. Conversely for spin with $m_S = -1/2$ (β).

Spin-Spin Interactions - Hyperfine

For the interaction between an electron and a nucleus

$E_{hyperfine} = +m_s m_I A$ A = hyperfine coupling constant (compare with NMR's J)

If the nuclear spin is aligned with the field, then the total field that the electron feels is larger. This results in a relative destabilization of an electron which is also aligned with the field. In other words, if the electron and nuclear spins are both α (or both β), then the energy of the system *increases* (opposite to NMR). However, if the spins are aligned opposite to each other, the energy of the system is *decreased* relative to the energy for no interaction.

This yields the following for the total energy of the system

$E_{Zeeman} = -m_{I_A} g_{N_A} \beta_N H - m_{I_B} g_{N_B} \beta_N H - m_{I_A} m_{I_B} J_{AB}$

Environmental Differences - g value

When we talk about local fields effecting the total field that a local electron feels, we refer to variations in g value. This is effectively like chemical shift in NMR. The difference is that variations in g value usually arise from orbital effects, rather than environment. Organic radicals are simple systems and the g value is very near that of the free electron (g_e). For metals, however, interactions between the magnetic moment of the electron and the orbital angular momentum of the electron can lead to large variations in g value.

g-anisotropy

Since g values in metals arises from interactions with the orbital angular momentum, it is not surprising that this interaction varies with the angle between the applied field and the electronic orbital of interest (for electronic orbitals above the fully symmetric s orbital). This is refered to g-anisotropy.

applications

radicals and metal centers in proteins
nitroxide radicals monitor motion