Lecture 14: Analysis of Cooperative Binding

Goals:

- Relate distribution of bound ligands to degree of cooperativity
- Understand approximation that gives the Hill equation.
- Generate Hill Plot, obtain K_D and n_h from Hill plot,
- Interpret n_h in terms of type of cooperativity.

Review of Types of Binding:

Non-cooperative: No interaction between sites. A protein with a single site must show non-cooperative binding.

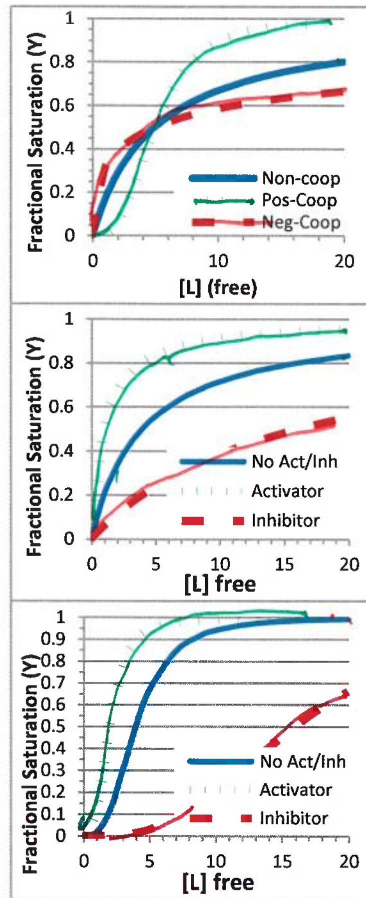
Homotropic positive cooperativity: Multiple interacting ligand binding sites required, binding at one increases affinity at another by increasing R state.

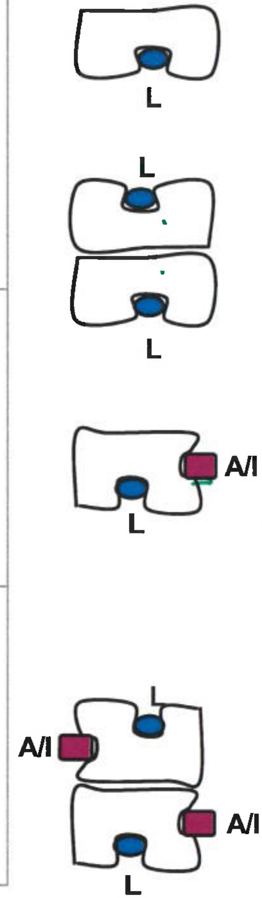
Homotropic negative cooperativity: Multiple interacting ligand binding sites required, binding at one decreases affinity at another by increasing T state.

Allosteric control - non-cooperative binding:

- Heterotropic activator increases R-state. Binding affinity of ligand for one or more non-interacting sites increases.
- Heterotropic inhibitor increases T-state. Binding affinity of ligand for one or more non-interacting sites decreases.

Allosteric control with cooperative binding: Heterotropic activator increases R-state, increasing average affinity. Heterotropic inhibitor increases T-state, reducing average affinity. Ligand binds to multiple interacting states (homotropic) with some form of cooperative binding, neg or positive (positive cooperativity for the **ligand** is shown here).





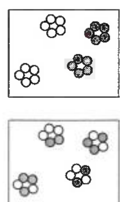
Binding of N-Ligands:

Non-cooperative Binding (regardless of # sites): $Y = \frac{[L]}{K_D+[L]} = \frac{[L]^1}{K_D+[L]^1}$ *eq. [1]*

Y=0.5 when $[L]=K_D$

Infinitely Positive Cooperativity - *the binding of one ligand makes the binding of others very favorable, only [M] and [ML$_n$] are seen. For n-binding sites:* $Y = \frac{[L]^n}{K_{D1}K_{D2}\cdots K_{Dn}+[L]^n}$ *eq. [2]*

Y = 0.5 when $[L] = \sqrt[n]{K_{D1}K_{D2}\cdots K_{Dn}}$



Cooperative systems – General equation (Hill Equation): $Y \approx \frac{[L]^{n_h}}{K_{D-ave}^{n_h}+[L]^{n_h}}$ *eq. [3]*

Y= 0.5 when [L] = $K_{D\text{-}Ave}$

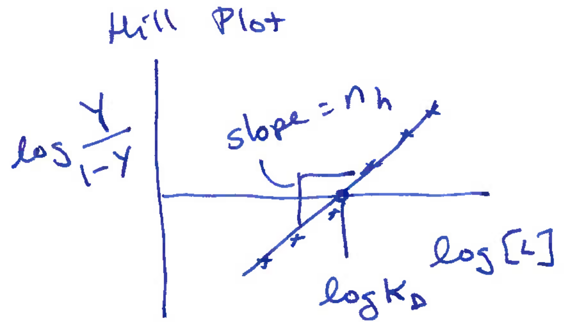
n_h is the Hill coefficient. The power that [L] is raised to.

Reflection: *What would you predict for the Hill coefficient for the following:*

1. Non-cooperative binding (eq [1])? 1
3. Infinitely strong positive cooperativity (eq [2])? n (# sites)
2. Positively cooperative binding (eq [3])? $1 < n_h < n$
4. Negative cooperativity? $0 < n_h < 1$

The Hill plot is based on a transformation of eq. [3].

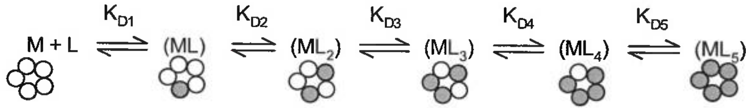
$$\log\left(\frac{Y}{(1-Y)}\right) = \log\left[\frac{1}{K_{D-ave}}\right]^{n_h} + n_h \log[L]$$

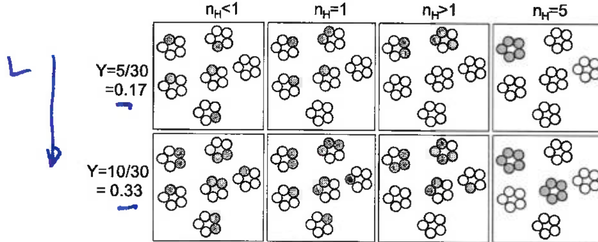


Plot of *log* [Y/(1 - Y)] versus *log*[L]

- The Hill coefficient, n_h, is the slope as the line crosses the *x*-axis.
- The $\log K_{D\text{-}ave}$ is the intersection of the Hill curve with the *x*-axis. This is the ligand concentration to ½ saturate the binding sites.

Example: Pentameric Cooperative Systems with different degrees of cooperativity.





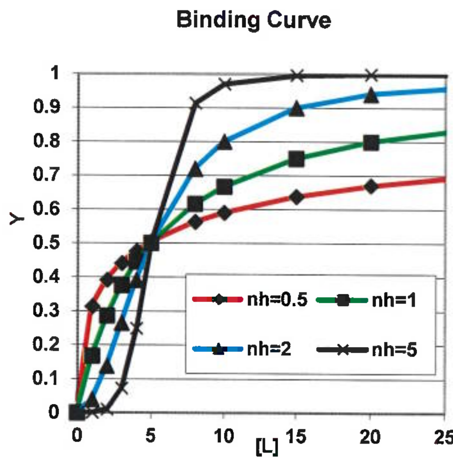
How does the distribution of bound ligands change with the degree of cooperativity?

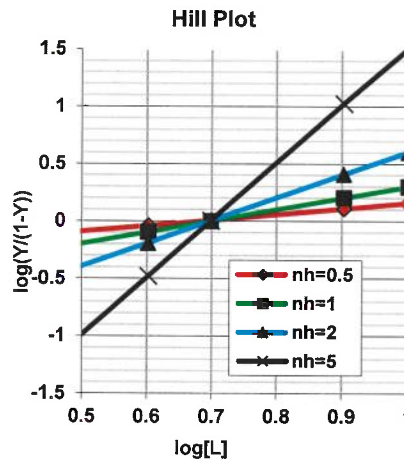
Neg coop: See lower occupancy

Non-coop: Random

Pos-coop: See intermediates, ML4, ML3

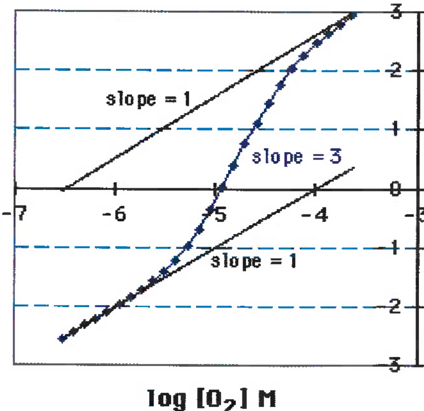
∞ pos-coop: M or ML5





Hill Plot for Human hemoglobin:

Low ligand: At very low ligand concentration, the binding *appears* non-cooperative because most of the macromolecule is in the [M] form, other forms (e.g. [ML]) are not found. Therefore, the Hill plot is initially linear, with a slope =1, intersecting x-axis at $\log K_{D1}$.



High ligand: At very high ligand concentration, the binding also *appears* non-cooperative because most of the macromolecule is in the [ML_3] form and only one ligand can bind. Therefore, the Hill plot is again linear, with a slope = 1, intersecting the x-axis at log K_{D4}.

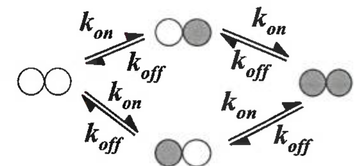
It is difficult to obtain data at very low and very high [L], so only the central part of the Hill plot is usually obtained.

Lecture 14-B: Microscopic and Macroscopic Binding Constants:

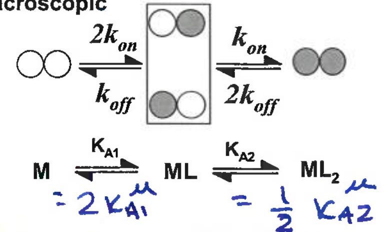
Microscopic K_A (K_A^{μ}): This is the association constant for a *single site*, and is just the ratio of the on- and off-rates: $K_A^{\mu} = k_{ON}/k_{OFF}$. It reflects the *intrinsic* affinity between the protein and the ligand.

$\Delta G^{o} = -RT \ln K_A^{\mu}$.

This is what would be measured for a single distinct binding site. *If K_A^{μ} changes from one binding step to another, the system is cooperative:*



- **Non-cooperative:** K_A^{μ} is the same for each step.
- **Positive cooperativity:** K_A^{μ} increases (K_D^{μ} decreases)
- **Negative cooperativity:** K_A^{μ} decreases (K_D^{μ} increases)

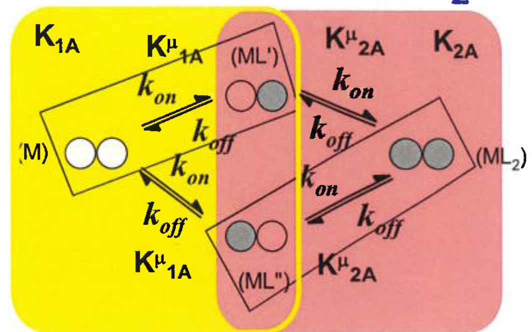


Macroscopic K_A: This is the *observed* K_A based on the experimental measurement of the concentrations of the various species, i.e. $K_{A1}=[ML]/[M][L]$.

The macroscopic K_A values are related to the microscopic ones by statistical factors:

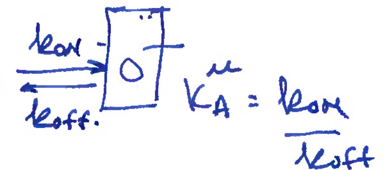
A) Statistical factors from concentrations.

For a dimeric system, there are two possible intermediates where one ligand is bound. They are labeled ML' and ML" in the diagram on the right. These are indistinguishable from each other by experimental measurement and equal in concentration. The microscopic and macroscopic binding constants for both steps are:



$$K_{1A}^{\mu} = \frac{[ML']}{[M][L]} = \frac{[ML"]}{[M][L]}$$

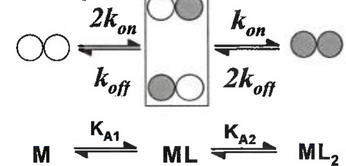
$$K_{1A} = \frac{[ML]}{[M][L]} = \frac{([ML']+[ML"])}{[M][L]} = \frac{[ML']}{[M][L]} + \frac{[ML"]}{[M][L]} = K_{1A}^{\mu} + K_{1A}^{\mu} = 2 \times K_{1A}^{\mu}$$



$$K_{2A}^{\mu} = \frac{[ML_2]}{[ML'][L]} = \frac{[ML_2]}{[ML"][L]}$$

$$K_{2A} = \frac{[ML_2]}{[ML][L]} = \frac{[ML_2]}{([ML']+[ML"])[L]} = \frac{[ML_2]}{2[ML'][L]} = \frac{1}{2} K_{2A}^{\mu}$$

B) Statistical factors from kinetic-rates.



For any reaction, the equilibrium constant is: $K_A = \frac{k_{on}^{Total}}{k_{off}^{Total}}$

The microscopic binding constants are just: $K_{A1}^{\mu} = k_{on\text{-}1}/k_{off\text{-}1}$.

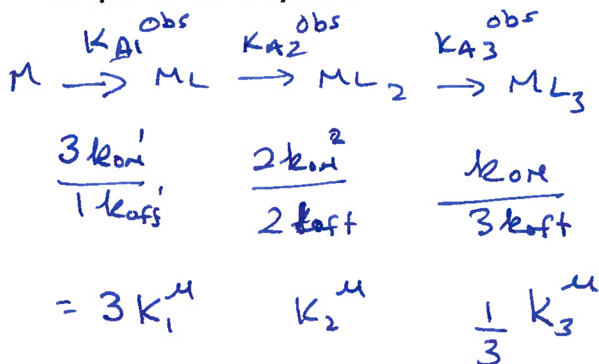
For the first binding event: $K_{A1} = \frac{k_{on}^{Total}}{k_{off}^{Total}} = \frac{2k_{on}}{k_{off}} = 2 \times K_A^{\mu}$

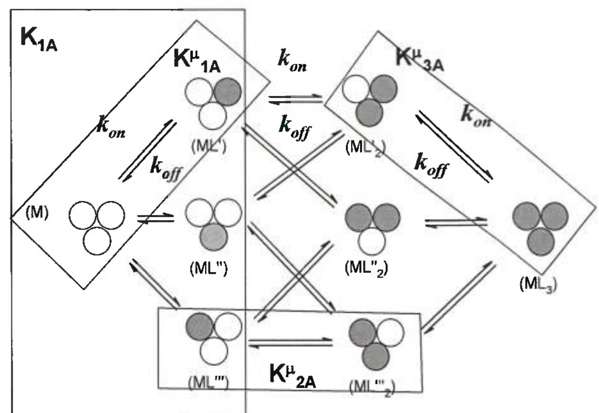
(There are two ways to form the [ML] species, but only one way for the ligand to leave.)

For the second binding event: $K_{A2} = \frac{k_{on}^{Total}}{k_{off}^{Total}} = \frac{k_{on}}{2k_{off}} = \frac{1}{2} \times K_A^{\mu}$

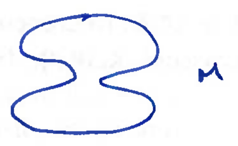
(There is only one way for the second ligand to bind, but there are two ways for the ligand to leave.)

Example – Trimeric System:



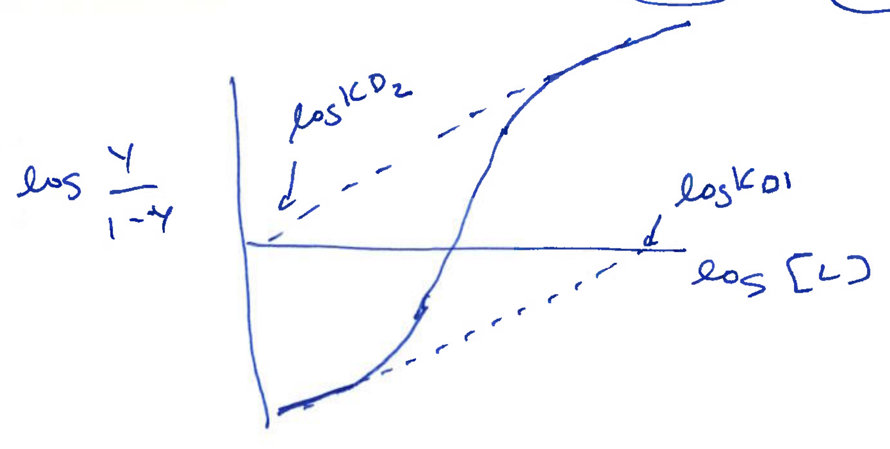


$$\Delta G^{o}_{bind} = -RT \ln K_A^{\mu}$$



$$K_{A1}^{MACRO} = \frac{[ML]}{[M][L]}$$

K_{A2}^{MACRO}

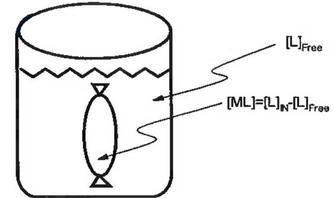


Summary of Ligand Binding:

- Y = Fractional saturation. From 0 to 1.
- Y = [ML]/([ML]+[M]) (one site).
- *n* = Number of binding sites.
- K_A = Association (binding) equilibrium constant, K_A=[ML]/[M][L]. M+L ⇌ ML
- K_D = Dissociation constant, K_D=1/K_A. Y = 0.5 when [L]=K_D, ML ⇌ M+L
- $K_{D\text{-}OBS}$ = Observed K_D for coop binding, [L] to give Y=0.5. $K_{D\text{-}OBS}$=*f*(K_{D1},K_{D2}...), *e.g. two sites:* $K_{D-OBS} = \sqrt[2]{K_{D1} * K_{D2}}$
- n_h = Hill coefficient, measure of cooperativity, maximum value is n (inf pos coop)

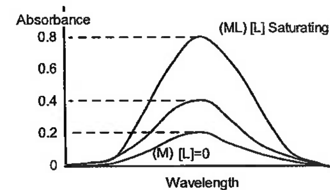
How to Measure Y:

i) Equilibrium dialysis.



- Protein (M_T) inside dialysis bag, cannot leave (semi-permeable).
- Add ligand to outside, after equilibrium is reached, L_{IN} = [ML] + L_{Out}.
- Y = (ML)/(M_T) = (L_{IN}-L_{OUT})/M_T, for a ligand concentration of L_{OUT}.

ii) Spectrophotometric.

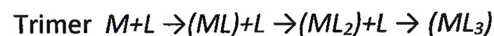


- Measure absorption when [L]=0, this gives A_M
- Measure absorption with saturating concentrations of [L], this gives A_{ML}.
- Vary [L], measure A
- Y = (A-A_M)/(A_{ML} – A_M)

Type & degree of cooperativity:

- n_h=1 for non-cooperative binding. *No* interaction between binding sites.
- n_h>1 for positive cooperativity: Binding of the 1st ligand *enhances* the binding of additional ones.
- n_h<1 for negative cooperativity: Binding of the 1st ligand *impairs* the binding of additional ones.
- The closer n_h is to n, the stronger the cooperativity, maximum value is n, # of sites.

Data Analysis--How to obtain K_D and Hill coef.

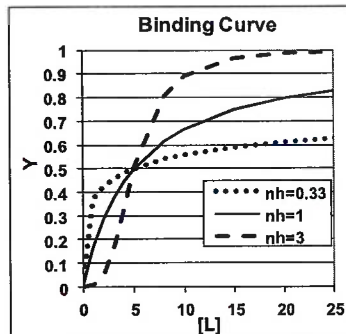

Trimer *M+L →(ML)+L →(ML₂)+L → (ML₃)*

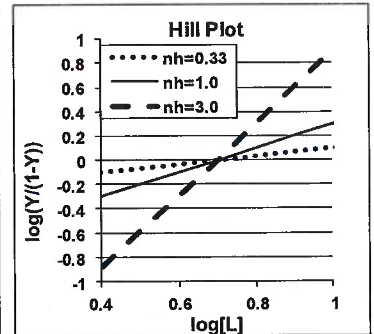
i) Binding Curve: *Plot Y versus [L].*

- K_D is [L] to give Y=0.5. This is the true K_D for non-cooperative binding, $K_{D\text{-}OBS}$ for cooperative binding.

ii) Hill Plot: Plot log(Y/(1-Y)) *versus* log[L]

- K_D - Ligand concentration when curve crosses x-axis (Y=0.5). This is the true K_D for non-cooperative binding, $K_{D\text{-}OBS}$ for cooperative binding.
- ***n_h***: Slope, Δ(log(Y/(1-Y))/Δlog([L]), *when curve crosses x-axis.*





$$Y = \frac{[L]}{K_D + [L]} \qquad Y = \frac{[L]^{n_h}}{K_{D\text{-OBS}}^{n_h} + [L]^{n_h}} \qquad Y = \frac{[L]^3}{K_{D1}K_{D2}K_{D3} + [L]^3}$$

iii) Distribution of bound ligands:

Low [L]:

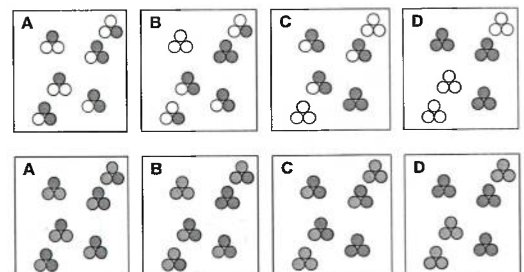
A: Negative cooperativity – tend to see just (M) and (ML)

B: Non-cooperative: Random distribution (M), (ML), (ML_2), etc.

C: Pos-cooperative – tend to see more (ML_2) and (ML_3)

D: Infinitely positive cooperative – only see (M) and (ML_n)

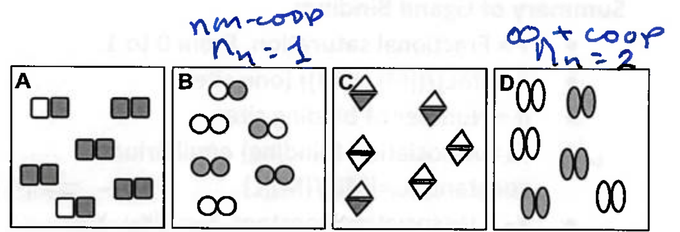
High [L]: All can be saturated if [L] >> K_D.



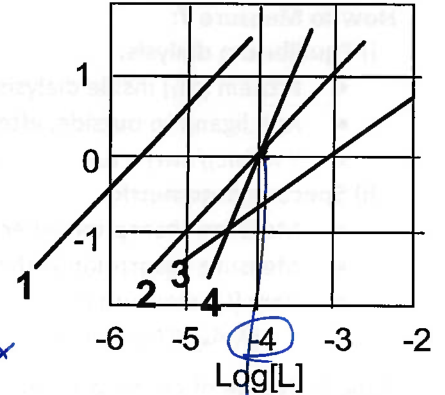
Important parameters and how to obtain them:

Parameter	One-site or *n*-sites non-cooperative	Cooperative
K_D (This is always [L] that gives Y=0.5)	1. Binding Curve, Y=0.5, [L] = K_D 2. Hill Plot, x-intercept= log K_D.	1. Y = 0.5 on binding curve, [L]=$K_{D\text{-}OBS}$ 2. *x*-intercept of Hill Plot = log $K_{D\text{-}OBS}$
n_h (Hill coefficient)	=1	Slope of Hill plot when Y=0.5 (*log[Y/[1-Y]] = 0)*
ΔG°	ΔG° = -RT ln K_A = -RT ln (1/K_D)	**ΔG° ≠ -RT ln (1/$K_{D\text{-}OBS}$)**

Practice Problems:



1. You are measuring the binding of a ligand to four different dimeric proteins. You have a magic camera that allows you to take a snapshot of the distribution of bound ligands at equilibrium. In all cases the ligand concentration is 10^{-4} **Molar**. Free ligand is not shown and subunits with ligand bound are shaded. You will find it useful to determine Y for these four cases, a column has been provided on the table to enter these values.

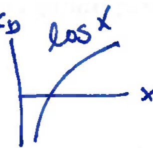


i) Match the distribution of bound ligands to the curve on the Hill plot and indicate the correct match in the table below.

Protein	Hill Curve (1-4)	Y
A	1	5/6 = 0.83
B	2	= 0.5
C	3	= 0.25
D	4	= 0.5

— lowest K_D

-highest K_D

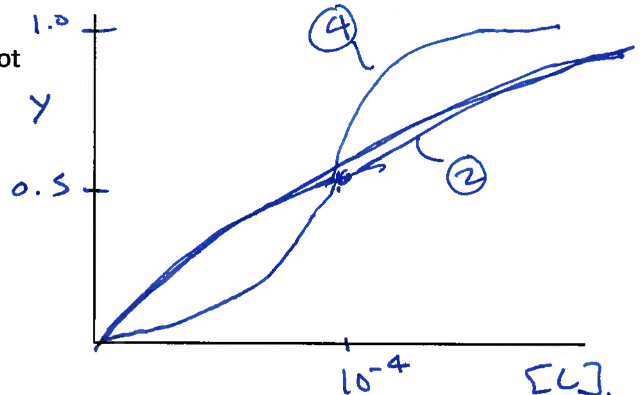


$\log K_D = -4$

$10^{\log K_D} = 10^{-4} = K_D$

ii) Sketch the binding curve (Y versus [L]) that you would expect to see for the proteins that generated the Hill plot curves labeled 2 and 4. Use the graph on the right. Be sure to label the axis and provide a scale.

2. non.



2. The Hill plot on the right shows the effect of BPG on the binding of oxygen to hemoglobin. Answer the following questions.

i) Is BPG an allosteric activator or inhibitor? Why?

↑ BPG ↑ K_D ∴ inhibitor

ii) What effect does BPG have on the cooperativity of oxygen binding? Does it increase it or decrease it?

iii) How does the change in cooperativity enhance O_2 delivery?

Hb - Oxygen Binding