

Ligand Binding: The Determination of K_d .

We cannot over *EMPHASIZE* that this is perhaps the MOST important experiment we will do ALL semester!

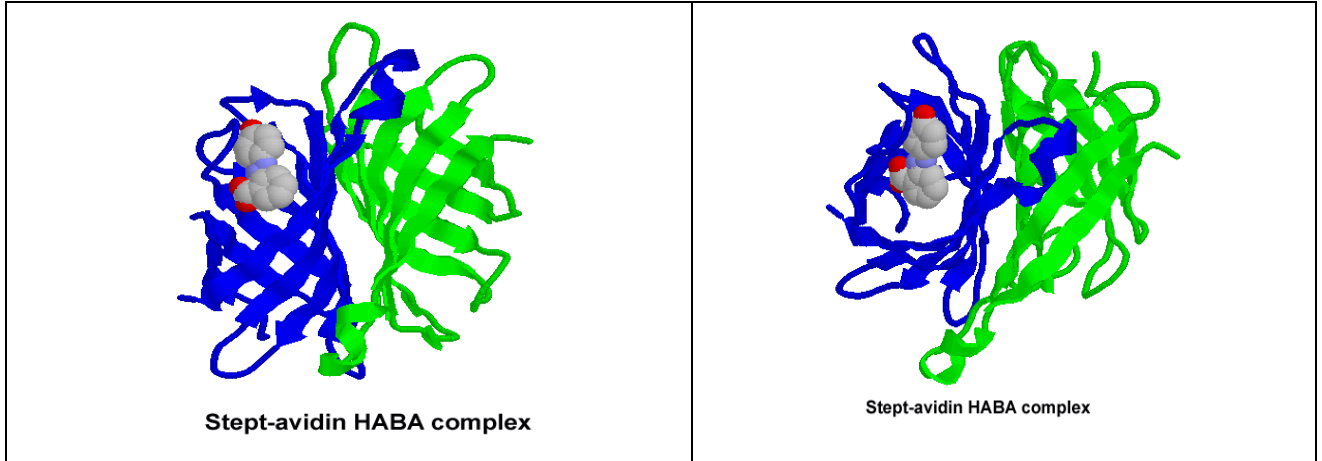
THE PURPOSE OF ALL LIGAND BINDING STUDIES IS TO DETERMINE K_d for the interaction of a ligand with a receptor or protein!!!!

Ligand Binding is the basis for ALL protein (and enzymatic) molecular recognition and specificity!

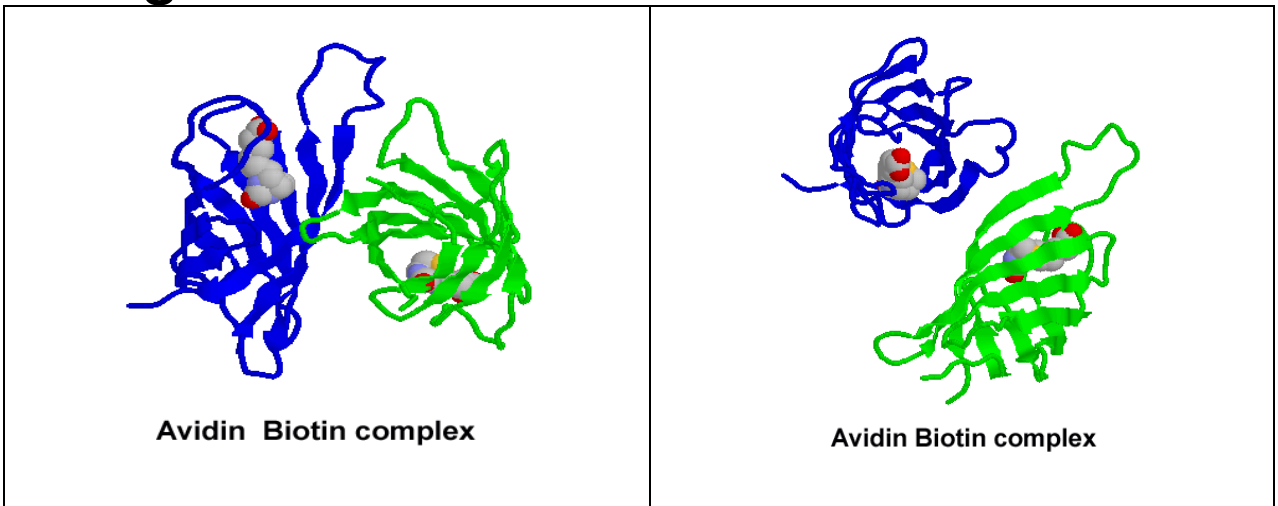
Examples:

- signal transduction pathways.
- membrane transport.
- binding of gaseous ligands.
- allosteric regulators.
- enzymatic competitive inhibitors.
- interaction of many drugs with receptors.
- DNA binding proteins:DNA interactions.
- protein:protein interactions.

First, we will determine the K_d for the binding of HABA to egg white avidin:

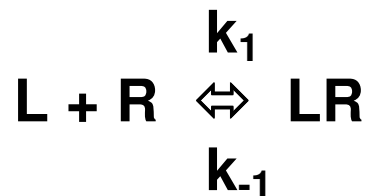


Then by a competitive ligand binding experiment, determine the K_d for imino-biotin binding to avidin.



Important: Ligands ARE NOT substrates! *But the binding of a substrate can be thought of as a ligand binding process.*

Starting Premise: You are studying a system at EQUILIBRIUM.



At EQUILIBRIUM means:

rate of forward rxn = rate of reverse rxn

$$v_f = k_1[\text{L}][\text{R}] = k_{-1}[\text{LR}] = v_r$$

collecting terms:

$$\frac{[\text{L}][\text{R}]}{[\text{LR}]} = \frac{k_{-1}}{k_1} = K_d$$

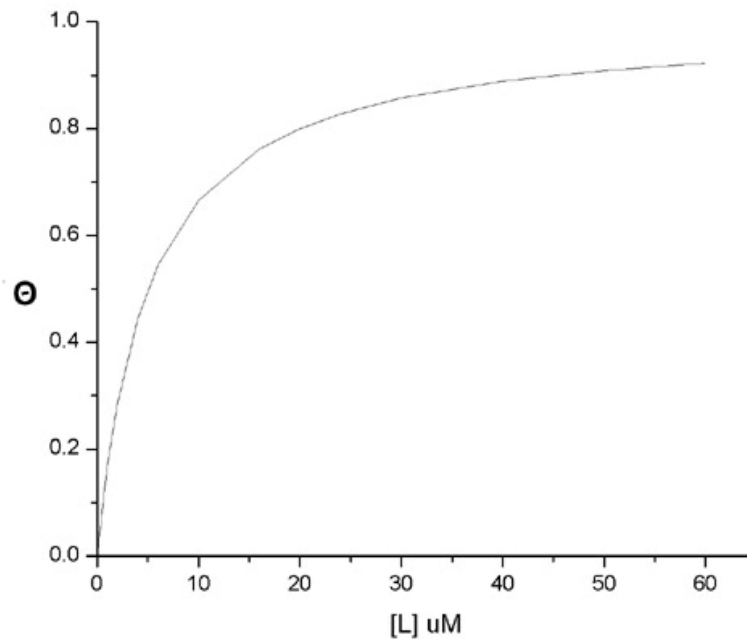
Thus, K_d has two definitions and ways to determine:

- thermodynamic (i.e., conc of L, R, and LR).
- kinetic (determine k_1 and k_{-1}).

Thermodynamic Treatment: from above eqn:

$$K_d = \frac{[L][R]}{[LR]}$$

Let Θ = fraction of bound receptors
$$\Theta = \frac{[LR]}{[LR] + [R]} = \frac{[LR]}{[R_t]} = \frac{[L]}{K_d + [L]}$$



when $K_d = [L]$ then $\Theta = 0.5$. This is a **3rd definition** of K_d ! The problem with this treatment is determining $[L]$ and Θ (see below).

**A more practical approach determine [LR]
starting with:**

$$\Theta = \frac{[LR]}{[R_t]} = \frac{[L]}{K_d + [L]}$$

[LR] is usually the thing you observe and [L] is usually calculated knowing [L_{total}] and [LR]! So solving the above eqn for [LR]:

$$[LR] = \frac{[R_t][L]}{K_d + [L]}$$

This is an equation for a square hyperbola where:

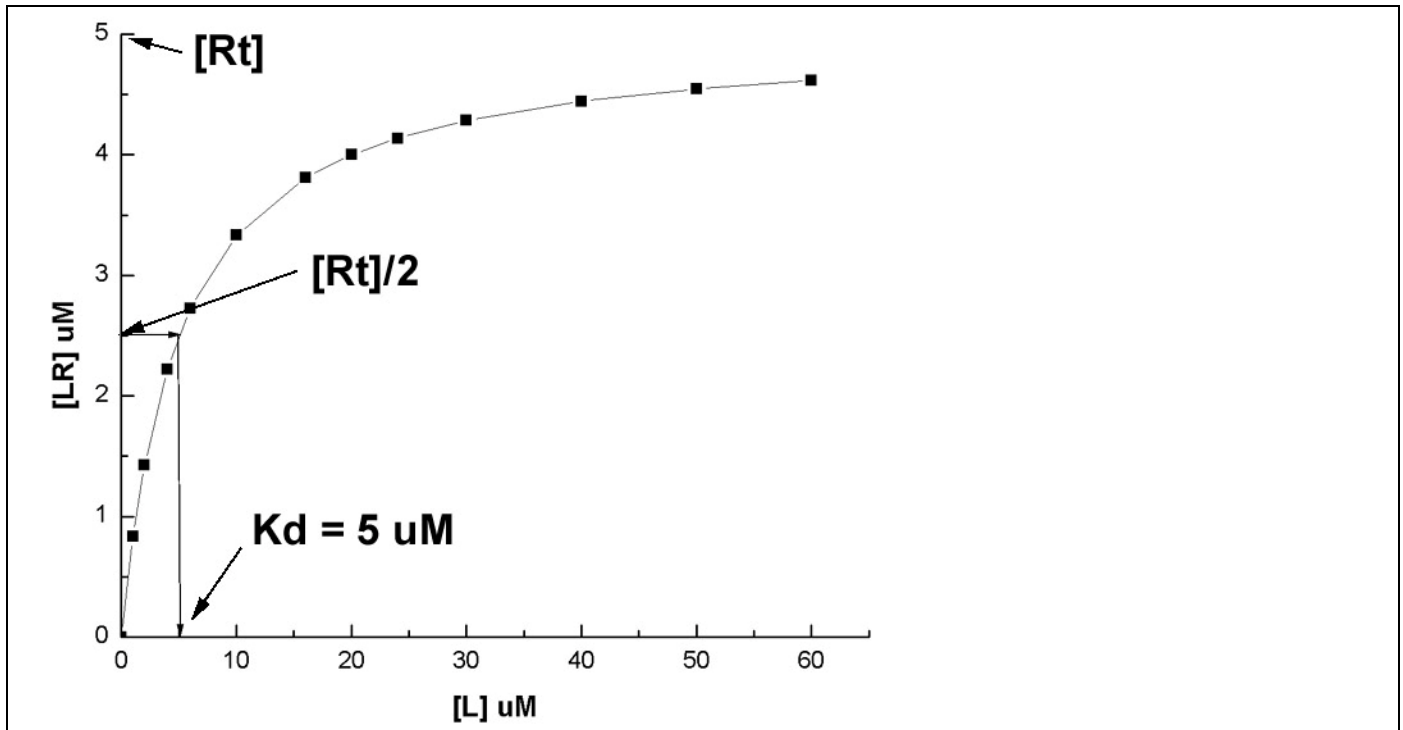
- **[R_t] and K_d are constants.**
- **[LR] is observed.**
- **[L] is calculated.**

Consider three conditions:

- **when [L] >> K_d, then [LR] → _____?**
- **when [L] = K_d, then [LR] = _____?**

This second condition provides us with a third empirical definition for K_d!!!!

“ K_d is the $[L]$ that makes $[LR] = [R_t]/2$ ”



Note: In order to analyze this data you MUST:

- first determine $[R_t]$.
- then determine $[R_t]/2$.
- finally, determine K_d .

To reiterate, the THREE definitions of K_d :

1. $K_d = [L][R]/[LR]$
2. $K_d = k_{-1}/k_1$
3. K_d is the $[L]$ that makes $[LR] = [R_t]/2$

In this experiment we will do a direct spectrophotometric titration of egg white Avidin (R) using a ligand (L) called HABA (Hydroxybenzene Azo Benzoic Acid).

- You will measure the HABA bound to the protein receptors (i.e., LR) by the change in Absorbance at 500 nm.**
- Knowing $[L_t]$ added, you calculate $[L]$ ($[L] = [L_t] - [LR]$).**
- Plot $[LR]$ vs. $[L]$.**
- Extrapolate your data to give $[R_t]$, and “Voila” you can determine K_d ! Sounds simple doesn't it?**

From a historical perspective:

- Today, hyperbolic data can easily be fit to a hyperbolic equation with appropriate software and a PC (or Mac).**
- But in pre-computer historical times things were not that simple since it is impossible to extrapolate to infinite $[L]$!**
- Therefore people transformed their non-linear data into linear forms by various mathematical “tricks”.**

The Double Reciprocal Plot

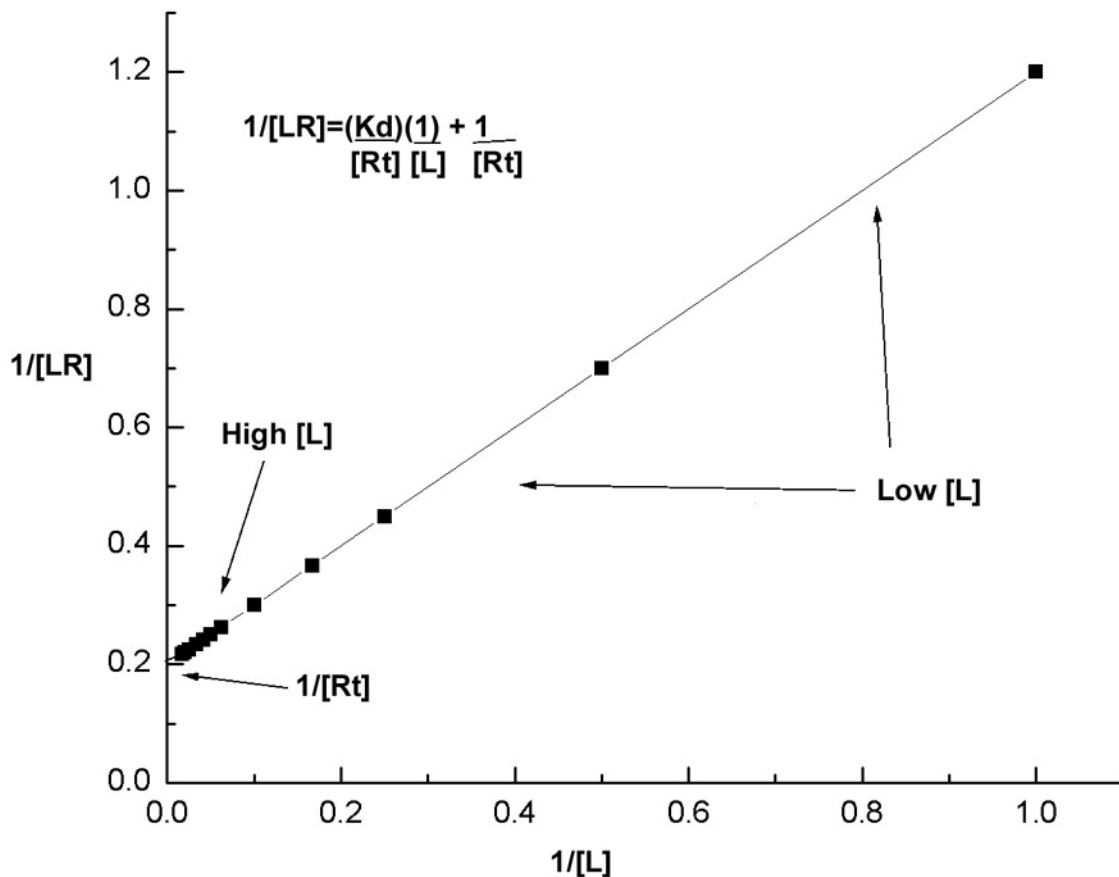
Starting with:

$$[LR] = \frac{[R_t][L]}{K_d + [L]}$$

Take inverse of both sides:

$$\frac{1}{[LR]} = \frac{(K_d)(1)}{(R_t)([L])} + \frac{(1)}{[R_t]}$$

which is a linear eqn of the form
 $y = mx + b$



Without the aid of a computer, it is much easier to extrapolate the data to $[R_t]$ in a linear plot, which is necessary to determine K_d .

Often, such ideal plots are difficult, if not impossible, to obtain due to error in making the measurement of $[LR]$, especially at low $[L]$ values. However, if you remember that you have to first determine $[R_t]$, then this should aid in analysis!

Scatchard Plot:

Again, starting with:

$$[LR] = \frac{[R_t][L]}{K_d + [L]}$$

the linear transform is:

$$\frac{[LR]}{[L]} = \frac{[R_t]}{K_d} - \frac{[LR]}{K_d}$$

Plot $[LR]/[L]$ vs. $[LR]$, the slope = $-(1/K_d)$ and the X-intercept is $[R_t]$. (See NB&B for a Scatchard Plot).

You might be asking, why is it necessary to empirically determine $[R_t]$ if you know how much R you added?

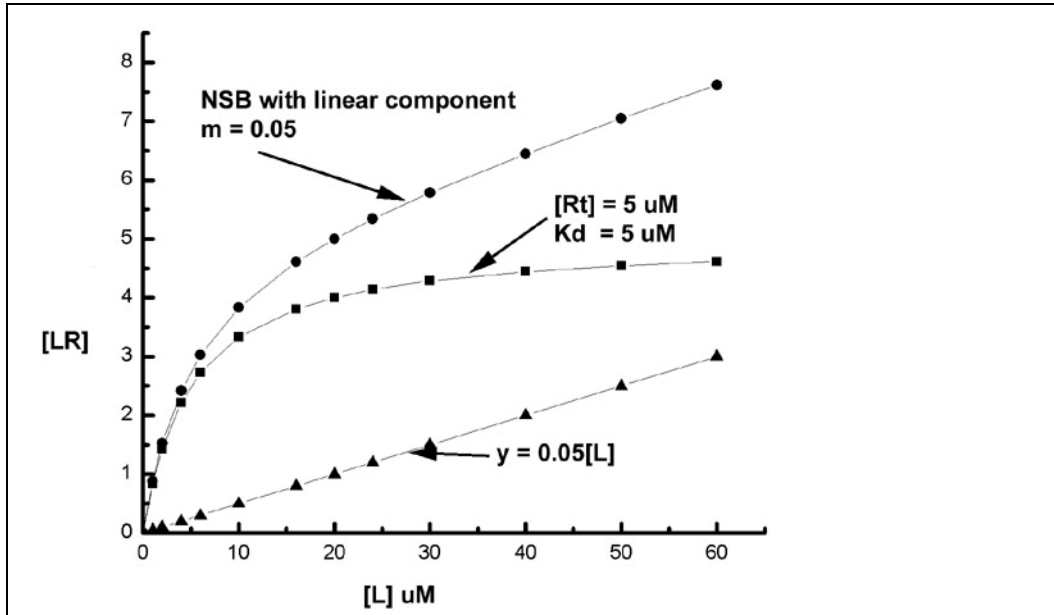
Sometimes (especially with membrane receptors) you can only guess how much R is present at the outset, i.e. get within a ballpark number. However in order to determine K_d you have to know the empirical observed value quantitatively!

Non-Specific Binding (NSB): A very real complication.

In this experiment, the spectral properties of the ligand change when it binds to the receptor. How is this related to the “solvent” environment of the ligand?

Suppose the ligand can bind NON-SPECIFICALLY to the protein, giving rise to similar spectral changes.

- which binding process would you expect to be “tighter”?**
- would you expect to see saturation with NSB as you do with binding to R?**



Above figure was generated using:

$$[LR] = \frac{[R_t][L]}{K_d + [L]} + (0.05)[L]$$

- In this exaggerated data set, the linear component is quite obvious at high [L], where the R has been saturated.
- When NSB is observed it must be subtracted from the data in order to observe the hyperbolic behavior.

$$y_{\text{cor}} = y_{\text{obs}} - y_{\text{linear}}$$

$$[\text{LR}]_{\text{cor}} = [\text{LR}]_{\text{obs}} - [\text{LR}]_{\text{NSB}}$$

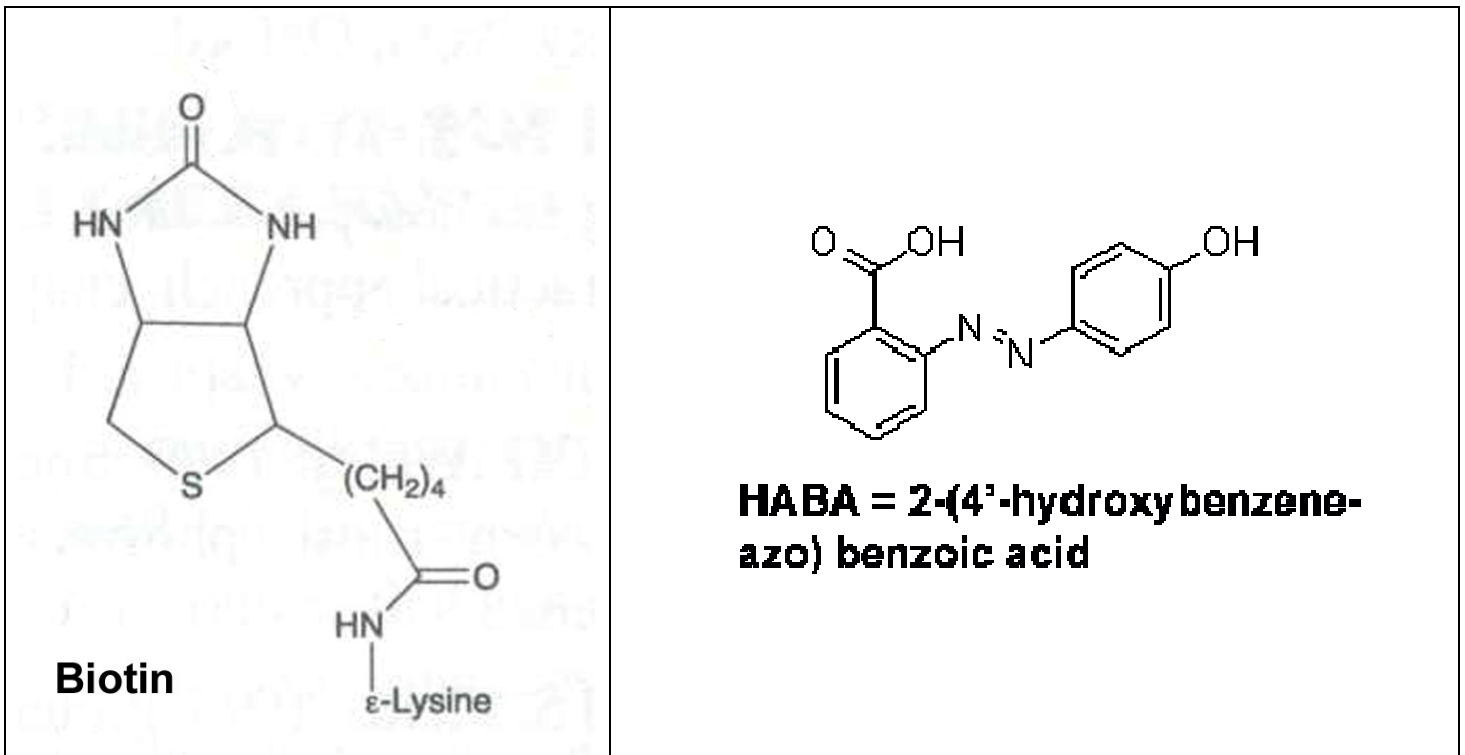
This can be done analytically (find slope of linear component) or graphically (old school method using a ruler!).

How would you know if you had “OVER” compensated for NSB? “Under” compensated?

Experimental Details:

Avidin: a homotetrameric protein with 4 receptors.

- naturally binds biotin.
- tetrameric mwt = 68 kD; monomeric = 17 kD.
- 1 receptor/monomer.
- receptors do not interact, no cooperativity.
- HABA is the ligand for which we will determine K_d .
- consult protocol for ϵ_{500} for HABA bound and unbound.



Designing Experiment:

1. Concentration of R: ideally you want $[R] < K_d$, however you do not know what is the value for K_d ! Initially, you can guess what you think K_d might be, say 10 μM . Now, choose a $[R] < 10 \mu\text{M}$.
2. You want to keep $[R_t]$ “*constant*” throughout experiment, however you are titrating (i.e. adding) L! Therefore, $\text{Vol}_{\text{total}}$ will change with each addition! Minimize volume change as much as possible ($<10\%$), using a fairly high stock [L].
3. But, in order to get a good determination of K_d you have to get some data points (at least 3 or 4) for $[L] < K_d$, so the concentration of stock L can not be **too** high (this requires some trial and error).
4. Get to high enough concentration of [L] in order to see the data become asymptotic with R_t , or in case of NSB, you can clearly resolve the linear component. So, $[L]_{\text{final}} = 5 - 6 \times K_d$.

5. Determine the volume of HABA necessary to satisfy #4, then decide how to aliquot it out!

I would strongly suggest you prepare a table like this in notebook before beginning experiment:

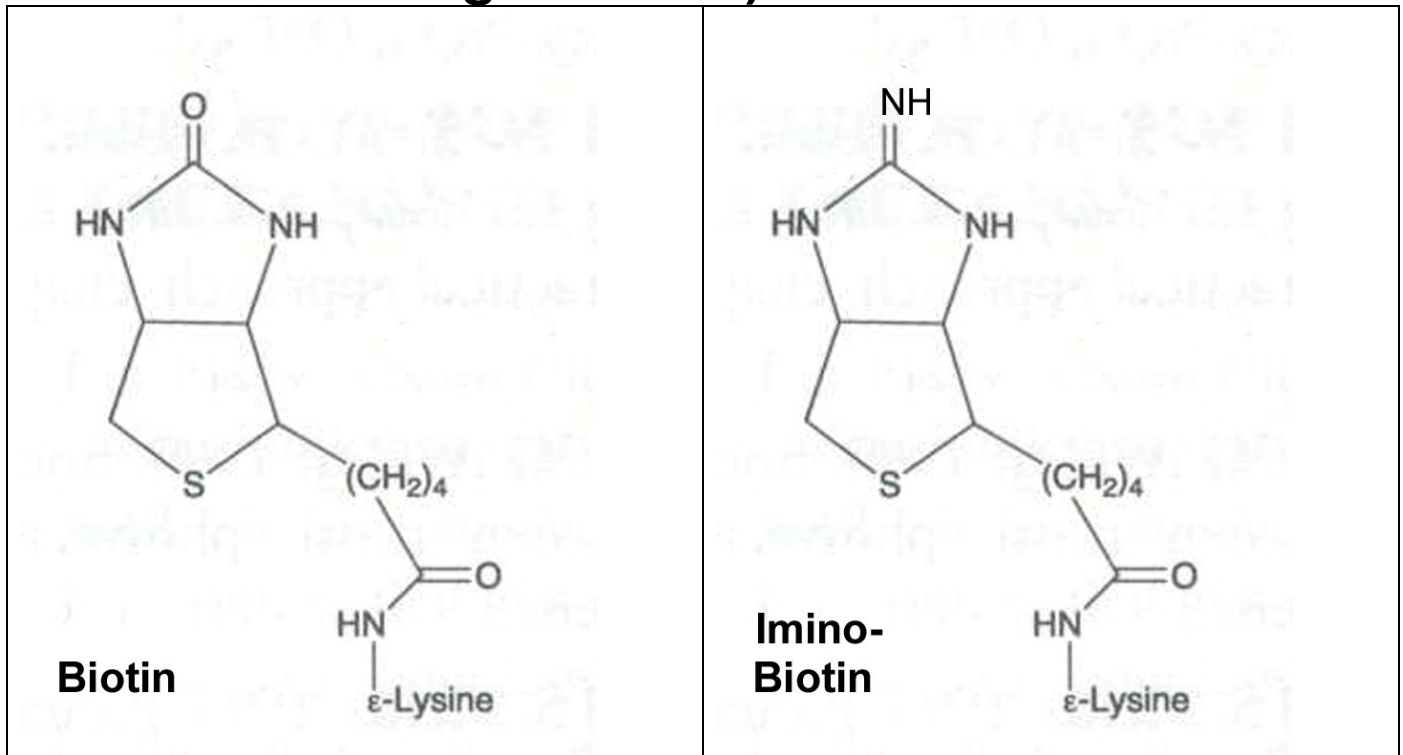
V Haba added	V_t HABA	[HABA]_t	A₅₀₀	[HABA] bound (= [LR])	[HABA] free (=[L])

In this type of experiment, book-keeping is very important!

As you collect data, open Excel and CONTINUOUSLY plot [HABA]_{bound} vs. [HABA]_{free} in order to monitor the quality of the data throughout experiment.

Competitive Ligand Binding Experiment

Having determined the K_d for the binding of HABA to avidin, we will now attempt to determine the K_d value for iminobiotin, IMB, (a structural analog of biotin).



The K_d for biotin is one of the lowest values known, therefore it is virtually impossible to determine $[Biotin]_{free}$ with each addition. The K_d value for IMB is higher making it better to use as a competing ligand.

$K_{L1,app}$ reflects not only K_{L1} , but the presence of L2 and how well it binds to R (i.e. K_{L2}).

Always, $K_{L1,app} > K_{L1}$ not because interaction of L1 with R has changed, but because of the presence of L2! Therefore, $[L1R]$ in presence of L2 will always $< [L1R]$ in absence of L2.

In a similar manner:

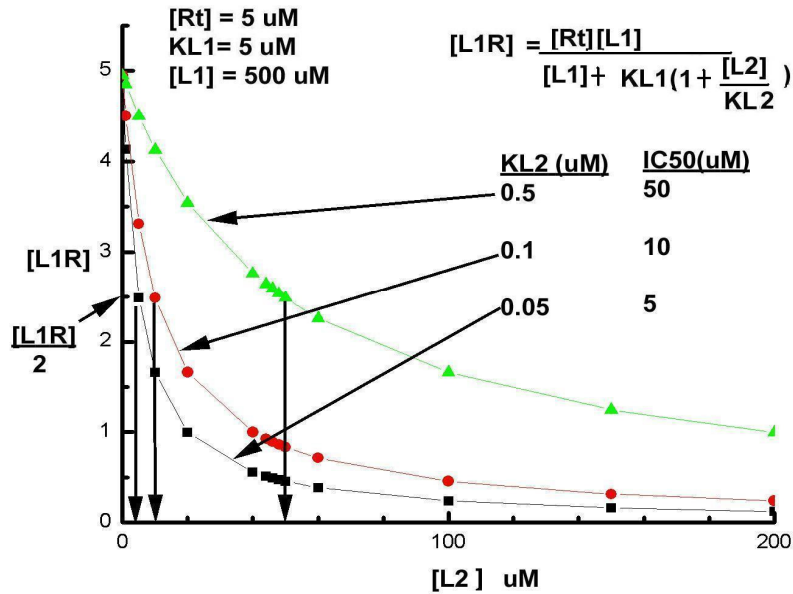
$$K_{L2,app} = K_{L2}(1 + ([L1]/K_{L1}))$$

The IC_{50} term:

This is a misnomer; however it is a commonly used term in competitive ligand binding literature. Literally, it is the concentration (C) of an “inhibitor” (I or L2) that gives a 50% decrease in the binding of L1 to the receptor.

$$IC_{50} = K_{L2,app}$$

So, IC_{50} is the $[L2]$ that causes a 50% decrease in $[L1R]$!



In order to accurately measure competition, what should $[L_1R]$ be relative to $[R_t]$ at the outset?

How is this accomplished?

Based on $[L_1R] = \frac{[R_t][L_1]}{K_d + [L_1]}$, you can calculate

the relationship between $[L_1]$, $[L_1R]$, and $[R_t]$ in absence of L2:

$[L_1]$	$[L_1R]$
$4K_d$	$0.8 R_t$
$9K_d$	$0.9 R_t$
$99K_d$	$0.99R_t$

- **So, in this experiment, we want to “poise” the system by having a vast excess of L1 (HABA) prior to addition of L2 (IMB).**
- **This poses a technical problem since there will be a lot of free HABA absorbing at 500 nm!**

Would it be better to use ABSOLUTE or DIFFERENCE spectroscopy to measure the loss of bound HABA due to addition of IMB? Why?