Reversible Ligand Binding Reactions:
Biological Significance and Analysis
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I. Biological significance of reversible ligand binding reactions

The dynamic chemistry of living organisms stems from their complex network of selectively-linked and highly-regulated chemical reactions, many of which involve reversible interactions between “receptors” and their “ligands.” A receptor (R) is generally considered to be a protein, enzyme, or other macromolecule whereas a ligand (L) is usually considered to be an ion, small molecule, substrate, or co-factor, but may also be another macromolecule, or “co-receptor”. Reversible receptor/ligand binding reactions are typically “weak” interactions between the receptor and ligand as they continuously and reversibly engage and disengage, over typically short time spans. Such interactions are ideal in terms of regulating biological processes because reaction reversibility allows biochemical processes to adjust rapidly and specifically to chemical fluctuations in the reaction environment.

Consider the now classic example of oxygen binding by hemoglobin, the oxygen transport protein of the blood. In arterial blood passing through the high oxygen environment of the lungs, each hemoglobin molecule, as carried in a red blood cell, tends to bind up to a maximum of four O₂ molecules. In venous blood passing through oxygen-depleted tissues, the oxygenated hemoglobin tends to release half of its bound O₂ thereby delivering O₂ to low oxygen environments where it can be consumed by mitochondria for energy production. The reversible binding and release of O₂ by hemoglobin repeats itself over and over as the blood continuously circulates through the body. This is a highly regulated process whereby several other hemoglobin ligands in the blood (e.g., H⁺ and Cl⁻ ions, 2,3-diphosphoglycerate, etc.) that reversibly bind hemoglobin in proportion to their concentrations and thereby altering or fine-tuning hemoglobin’s affinity for O₂.

The nuanced ligand binding properties of hemoglobin echo a recurrent theme found in contemporary studies of biochemical processes and their underlying reactions where molecular understanding of such processes hinges on the characterization and analysis of the associated reversible ligand binding reactions. The primary aim of this monograph is to forge a blueprint, as it were, for effective analysis of reversible ligand binding reactions. Topics are systematically ordered according to the increasing levels of reaction complexity. Simple monovalent receptor/ligand reactions (including weak acid/base interactions) are considered first, followed by the analysis of more complex multivalent receptor/ligand reactions in which case receptors have more than one binding site for the same ligand. Bivalent receptor/ligand reactions are analyzed in rigorous mathematical detail because exact solutions are possible and guidelines can easily be developed for determining whether more complex multivalent receptors bind their ligands non-cooperatively, anti-cooperatively, or cooperatively. By definition, non-cooperative ligand binding reactions describe receptors with all ligand binding sites having exactly the same affinity for ligand whether or not ligand already occupies other sites on the same receptor. In contrast anti-cooperative and cooperative ligand binding reactions describe receptors with ligand binding sites having with different affinities for ligand depending on whether or not ligand already occupies other sites on the same receptor. By definition, anti-cooperative ligand binding occurs when the ligand affinity of a multivalent receptor decreases when one or more of its ligand binding sites is already occupied. Conversely, cooperative ligand binding occurs when the ligand affinity of a multivalent receptor increases when one or more of its ligand binding sites is already occupied, as is the case for O₂ binding to hemoglobin, for example.

The main theme throughout the discussion here will be the application of reversible equilibrium chemistry for analyzing dynamic biological reactions that usually occur as irreversible, non-equilibrium, steady-state reactions in vivo. Although it might seem unlikely that such irreversible processes could be effectively analyzed according to the principles of reversible equilibrium chemistry, it is possible in some cases to gain deep insights into the non-equilibrium biological processes themselves, as illustrated by the selected examples discussed below. Most of the reversible equilibrium reactions discussed here will be considered both as dissociation and association reactions since biochemists (more so than chemists) are likely to consider reversible chemical reactions as running in both directions - e.g., ligand binding to receptors, substrate binding to but product dissociation from enzymes, etc. Obviously, for reversible equilibrium reactions, the thermodynamic process doesn’t change according the “direction” the reaction when written either as a dissociation or an association reaction. Conceptually, however, it
easier to discuss some reactions in terms of their dissociation constants \(K_{dn}\), such as acid/base dissociation reactions, while other reactions are more easily discussed in terms of their association constants \(K_{an}\), such as receptors/ligand reactions.

II. MONOVALENT equilibrium ligand binding reactions: Definitions and relationships

A monovalent receptor \((R)\) is a protein, macromolecule, enzyme, etc. with one specific binding site for ligand \((L)\) such as an ion, small molecule, co-receptor, etc.

Dissociation Reaction:
- Equilibrium dissociation reaction for a monovalent receptor: \([RL] \Leftrightarrow [R] + [L]\)
- Equilibrium dissociation constant: \(K_{dn} = [R][L]/[RL] = [L]_{50}\) \textit{Empirical definition}
- At \([L]_{50}\), \([R] = [RL]\) with 50% of the receptor binding sites occupied by ligand.
- \(pK_{dn} = -\log K_{dn} = -\log [L]_{50}\)
- Equilibrium fractional dissociation: \(Y_d = [R]/([R] + [RL]) = [R]/Co\) where \(Co = [R] + [RL]\);
- \(Y_d = K_{dn}/([L] + K_{dn}) = 1/(1 + 10^{pK_{dn} - pL}\text{), where } pL = -\log [L]\text{; and } [L] = 10^{-pL}\)

Association Reaction:
- Equilibrium association reaction for a monovalent receptor: \([R] + [L] \Leftrightarrow [RL]\)
- Equilibrium association constant: \(K_{an} = [RL]/[R][L] = 1/K_{dn} = 1/[L]_{50}\) \textit{Empirical definition}
- Equilibrium fractional association: \(Y_a = [RL]/([R] + [RL]) = [RL]/Co\)
- \(Y_a = [L]/([L] + K_{dn}) = 1/(1 + 10^{pL - pK_{dn}})\)

III. What are “weak” acids and bases as defined by the Brønsted concept?

In aqueous solutions, a “weak” acid or base act as receptor \((R)\) that reversibly binds a proton, which is the corresponding ligand \((L)\) of the reaction.
- According to the Brønsted concept, an acid is a “proton donor.” In contrast to a strong acid (e.g., HCl), which completely dissociates its H\(^+\) ions in an aqueous solution, a weak acid (e.g., CH\(_3\)COOH) dissociates only a small fraction of its bound H\(^+\) ions at equilibrium, unless additional base is present. In the presence of a strong or weak base (e.g., NaOH or CH\(_3\)NH\(_2\)), a weak acid dissociates a greater percentage of its bound H\(^+\) ions, which in effect, are “transferred” to the basic group in proportion to its concentration and its relative strength as a base.
- According to the Brønsted concept, a base is a “proton acceptor.” In contrast to a strong base (e.g., OH\(^-\)), which almost irreversibly binds any available H\(^+\) ions in an aqueous solution, a weak base (e.g., CH\(_3\)NH\(_2\)) only partially binds free H\(^+\) ions at equilibrium unless additional acid is present. In the presence of a strong or weak acid (e.g., HCl or CH\(_3\)COOH), OH\(^-\) ions generated by a weak base are neutralized by H\(^+\) ions from the acid depending its concentration and its relative strength as an acid.
A. Weak acid dissociation (ionization) in water

**Acid Dissociation Reaction:**

\[
\text{AH} + \text{H}_2\text{O} \rightleftharpoons \text{A}^- + \text{H}_3^+\text{O} \\
\text{weak acid} \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \cdot
B. Weak acid association (saturation) in water

**Acid Association Reaction:**

\[
\begin{array}{ccc}
A^- & + & H_3^+O \\
\text{weak acid conjugate base} & \text{weak acid proton acceptor} & \text{weak acid proton donor} \\
\text{weak base proton acceptor} & \text{weak base proton donor} & \text{H}_2O
\end{array}
\]

\[
K_{eq} = \frac{[A^-][H_3^+O]}{[A^-][H_3^+O]} = \frac{[A^-][55.5]/[A^-][H_3^+O]}{[H_2O]} = \frac{[H_2O]}{[H_2O]} = 55.5 M \quad \text{(assumed constant)}
\]

\[
K_{eq}/55.5 = K_{an} = \frac{[H_2O]}{[H_2O]} = \frac{[HA]/[A^-][H^+]}{= \text{equilibrium association constant}}
\]

**Summary:**

- Equilibrium association or saturation reaction: \([H^+]/[A^-] \rightleftharpoons [HA]\)
- Equilibrium association constant: \(K_{an} = [HA]/[H^+][A^-] = 1/K_{dn}\)
- \(K_{an} = 1/[H^+]_{50} = 1/K_{dn}\)
- \(pK_{dn} = -\log K_{dn} = -\log (1/K_{an}) = +\log K_{an}\)
- \(K_{dn} = 10^{-pK_{dn}}\)
- \(C_a = [A^-] + [HA] = \text{total concentration of ionized and unionized acid}\)
- Equilibrium fractional association, \(Y_d = [HA]/([A^-] + [HA]) = [HA]/C_a = 1 - Y_d\)
- \(Y_d = [H^+]/(K_{dn} + [H^+]) = 1/(1 + 10^{pH-pK_{dn}})\)

**Confusing concepts:**

- When \([H^+] \uparrow\) (increases), \(pH \downarrow\) (decreases).
- When \(K_{an} \uparrow\) (increases), \(K_{dn} \downarrow\) (decreases) & \(pK_{dn} \uparrow\) (increases) signifying stronger ligand binding!

**Example:** See Fig. 2 for graphs of \(Y_d\) vs. \([H^+]\) or \(pH\) for strong acid titration of acetate, \(CH_3COO^-\).

C. Weak base dissociation (ionization) in water

**Base Dissociation Reaction:**

\[
\begin{array}{ccc}
BH^+ & + & OH^- \\
\text{weak base conjugate acid} & \text{weak base proton acceptor} & \text{weak acid proton donor} \\
\text{strong base proton acceptor} & \text{proton donor} & \text{H}_2O
\end{array}
\]

\[
K_{eq} = \frac{[B][OH^-][BH^+]}{[B][55.5]/[OH^-][BH^+]} = \frac{[B][55.5]/[OH^-][BH^+]}{[H_2O]} = \frac{[H_2O]}{[H_2O]} = 55.5 M \quad \text{(assumed constant)}
\]

\[
K_{eq}/(55.5) = \frac{[B][OH^-][BH^+]}{[B][55.5]/[OH^-][BH^+]} = \frac{[B][55.5]/[OH^-][BH^+]}{[H_2O]} = \frac{[H_2O]}{[H_2O]} = 55.5 M \quad \text{(assumed constant)}
\]

\[
K_{eq}K_{w}/(55.5) = K_{dn} = [B][H^+]/[BH^+] = \text{equilibrium dissociation constant}
\]

**Summary:**

- Equilibrium dissociation or ionization reaction: \([BH^+] \rightleftharpoons [H^+] + [B]\)
- Equilibrium dissociation constant, \(K_{dn}\)
- \(K_{dn} = [H^+]/[BH^+] = [H^+]_{50}\)
- \(pK_{dn} = -\log K_{dn} = 10^{-pK_{dn}}\)
- \(K_{dn} = 10^{-pK_{dn}}\)
- \(pH = -\log [H^+]\)
- \([H^+] = 10^{-pH}\)
- \(C_b = [B] + [BH^+] = \text{total concentration of ionized and unionized base}\)
- Equilibrium fractional dissociation, \(Y_d:\)
- \(Y_d = [B]/([B] + [BH^+]) = [B]/C_b\)
- \(Y_d = K_{dn}/(K_{dn} + [H^+]) = 1/(1 + 10^{pK_{dn}-pH})\)

**Derive these equations for \(Y_d\) & \(K_{dn}\).**
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FIGURE 2: (A) Yd or Ya vs. [H\(^+\)]. (B) Ya vs. pH

### Reversible Ligand Binding Reactions

Yd = \([H^+]/(K_{dn} + [H^+])\)
Ya = \([H^+]/([H^+] + K_{dn})\)

### HCl Titration of Acetic Acid

Ac\(^-\) + H\(^+\) + Cl\(^-\) ⇌ HAc + Cl\(^-\)

Yd = \([Ac^-]/Co = K_{dn}/([H^+] + K_{dn})\)
Ya = \([HAc]/Co = [H^+]/([H^+] + K_{dn})\)

### NaOH Titration of Acetic Acid

HAc + Na\(^+\) + OH\(^-\) ⇌ Ac\(^-\) + Na\(^+\) + H\(_2\)O

Ya = \(1/(1 + 10^{(pH-pK_{dn})})\)

Confusing concepts:
- When \([H^+]\) \(\downarrow\) (decreases), pH \(\uparrow\) (increases).
- When K\(_{dn}\) \(\uparrow\) (increases), pK\(_{dn}\) \(\downarrow\) (decreases), thus signifying weaker ligand binding!

Example: Methylamine, CH\(_3\)NH\(_3^+\), ionization to CH\(_3\)NH\(_2\); pK\(_{dn}\) = 10.62

### D. Weak base association (saturation) in water

Base Association Reaction:

\[
\begin{align*}
B + H_2O & \rightleftharpoons BH^+ + OH^- \\
\text{weak base} & \text{proton acceptor} & \text{weak acid} & \text{proton donor}
\end{align*}
\]

K\(_{eq}\) = [OH\(^-\)][BH\(^+\)]/[B][H\(_2\)O] = [OH\(^-\)][BH\(^+\)]/[B][55.5] where [H\(_2\)O] = 55.5 M (assumed constant)

K\(_{eq} \times 55.5\) = [OH\(^-\)][BH\(^+\)]/[B] = K\(_w\) \times [BH\(^+\)]/[B][H\(^+\)] with K\(_w\) = [H\(^+\)][OH\(^-\)] = 10\(^{-14}\) = H\(_2\)O ionization constant.

K\(_{eq} \times 55.5\)/K\(_w\) = K\(_{an}\) = [BH\(^+\)]/[B][H\(^+\)] = equilibrium association constant

Summary:
- Equilibrium association or saturation reaction: \([H^+] + [B] \rightleftharpoons [BH^+]\)
- Equilibrium association constant: K\(_{an}\) = [BH\(^+\)]/[H\(^+\)][B]
- K\(_{an}\) = 1/[H\(^+\)]50 = 1/K\(_{dn}\)
- pK\(_{dn}\) = -\log K\(_{dn}\) = -\log (1/K\(_{an}\)) = log (K\(_{an}\))
- K\(_{dn}\) = 10\(^{-pK_{dn}}\)
- C\(_b\) = [B] + [BH\(^+\)] = total concentration of ionized and unionized base

Reversible Ligand Binding Reactions

- Equilibrium fractional association, \( Y_a \):
  \[ Y_a = [BH^+]/([B] + [BH^+]) = [BH^+]/C_B = 1 - Y_d \]
  \[ Y_a = [H^+]/(K_{dn} + [H^+]) = 1/(1 + 10^{pH-pK_{dn}}) \]

**Definition of \( Y_a \)**

**Derive these equations for \( Y_a \) & \( K_{dn} \)**

**Confusing concepts:**
- When \([H^+] \uparrow \) (increases), \( pH \downarrow \) (decreases).
- When \( K_{an} \uparrow \) (increases), \( K_{dn} \downarrow \) (decreases) & \( pK_{dn} \uparrow \) (increases) signifying stronger ligand binding!

**Example:** Methylamine, CH\(_3\)NH\(_2\), saturation to CH\(_3\)NH\(_3^+\); \( pK_{dn} = 10.62 \)

IV. *The Henderson-Hasselbalch equation and graphical representations*

Derivation of the Henderson-Hasselbalch equation for a monovalent acid:
- The equilibrium **dissociation constant** = \( K_{dn} = [H^+][A^-]/[HA] \)
- \(-\log K_{dn} = -\log [H^+] - \log ([A^-]/[HA]) = -\log [H^+] - \log (Y_d/Y_a)\)
- \( pK_{dn} = pH - \log ([A^-]/[HA]) = pH - \log (Y_d/Y_a) \)
- \( pH = pK_{dn} + \log ([A^-]/[HA]) = pK_{dn} + \log (Y_d/Y_a) = pK_{dn} + \log (Y_d/(1-Y_d)) \)

**Henderson-Hasselbalch equation**
- \( pK_{dn} = pH_{50} \) where \( Y_d = Y_a \) and \( \log (Y_d/Y_a) = \log (1) = 0 \)

**Example:** See Fig. 3 for graphs of \( pH \) vs. \( Y_d/Y_a \) for strong acid titration of acetate, CH\(_3\)COO\(^-\).

**FIGURE 3: Henderson-Hasselbalch equation for a monovalent acid. (A) \( pH \) vs. \( Y_d \). (B) \( pH \) vs. \( Y_d/(1-Y_d) \)**

NaOH Titration of Acetic Acid

\[ HAc + Na^+ + OH^- \rightleftharpoons Ac^- + Na^+ + H_2O \]

Henderson-Hasselbalch Equation

\[ pH = \log \left( \frac{Y_d}{1-Y_d} \right) + pK_{dn} \]

V. Determining the pH of solutions containing weak acids or weak bases

A. pH dependence on the concentration of a WEAK ACID in water

- Equilibrium dissociation or ionization reaction: [HA] ⇌ [H⁺] + [A⁻]
- Equilibrium dissociation constant: \( K_{dn} = [H^+][A^-]/[HA] \)
- If no other acid or base is present: \([H^+] = [A^-] \)
- For a weakly dissociating acid: \( C_a = [HA] + [A^-] \equiv [HA] \)
- \( K_{dn} = [H^+][H^+]/C_a = [H^+]^2/C_a \)
- \(-\log K_{dn} = pK_{dn} = -\log [H^+]^2 - \log (1/C_a) = 2 \text{pH} + \log C_a \)
- \( \text{pH} = (1/2) (pK_{dn} - \log C_a) \)

_Calculated dependence of pH, Yd, and Ya on C_a assuming pK_{dn} = 5.0._

<table>
<thead>
<tr>
<th>C_a (M)</th>
<th>pH</th>
<th>Yd</th>
<th>Ya</th>
<th>pK_{dn} = 5.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0</td>
<td>2.50</td>
<td>0.003</td>
<td>0.997</td>
<td>pH = (1/2) (pK_{dn} - \log C_a)</td>
</tr>
<tr>
<td>0.1</td>
<td>3.00</td>
<td>0.010</td>
<td>0.990</td>
<td>Yd = 1/(1 + 10^{pK_{dn}-pH})</td>
</tr>
<tr>
<td>0.01</td>
<td>3.50</td>
<td>0.031</td>
<td>0.969</td>
<td>Ya = 1/(1 + 10^{pH-pK_{dn}})</td>
</tr>
<tr>
<td>0.001</td>
<td>4.00</td>
<td>0.091</td>
<td>0.909</td>
<td></td>
</tr>
<tr>
<td>0.0001</td>
<td>4.50</td>
<td>0.240</td>
<td>0.760</td>
<td></td>
</tr>
<tr>
<td>0.00001</td>
<td>5.60</td>
<td>0.500</td>
<td>0.500</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>C_a (M)</th>
<th>pH</th>
<th>Yd</th>
<th>Ya</th>
<th>pK_{dn} = 5.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.00001</td>
<td>6.05</td>
<td>0.910</td>
<td>0.090</td>
<td>pH = (K_{dn}/2)^<em>(-1+(1+4</em>C_a/K_{dn})^{1/2})</td>
</tr>
<tr>
<td>0.00001</td>
<td>6.69</td>
<td>0.999</td>
<td>0.001</td>
<td>i.e. 2 x [H+] of water = 2 x 10^{-7}</td>
</tr>
</tbody>
</table>

B. pH dependence on the concentration of a WEAK BASE in water

- Equilibrium dissociation reaction: [NH₃⁺CH₃] + [OH⁻] ⇌ [H₂O] + [NH₂CH₃]
- Equilibrium dissociation constant = \( K_{dn} = [H^+][NH₂CH₃]/[NH₃⁺CH₃] \)
- For a weakly dissociating base: \( C_b = [NH₂CH₃] + [NH₃⁺CH₃] \equiv [NH₂CH₃] \)
- If no other acid or base are present: \([OH^-] \equiv [NH₃⁺CH₃] \equiv K_w/[H^+] \)
- \( K_w = 10^{-14} \), the water ionization constant.
- \( K_{dn} \equiv [H^+]^2C_b/K_w = [H^+]^2C_b/[K_w/[H^+] = [H^+]^2C_b/K_w \)
- \(-\log K_{dn} = pK_{dn} = -\log [H^+]^2 - \log C_b - \log (1/K_w) = 2 \text{pH} - \log C_b + \log K_w \)
- \( pK_{dn} = 2 \text{pH} - \log C_b - 14 \quad \text{pH} = (1/2) (pK_{dn} + \log C_b + 14) \)

_Calculated Dependence of pH, Yd, and Ya on C_b assuming pK_{dn} = 10.62._

<table>
<thead>
<tr>
<th>C_b (M)</th>
<th>pH</th>
<th>Yd</th>
<th>Ya</th>
<th>pK_{dn} = 10.62</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0</td>
<td>12.31</td>
<td>0.980</td>
<td>0.020</td>
<td>pH = (1/2) (pK_{dn} + log C_b +14)</td>
</tr>
<tr>
<td>0.1</td>
<td>11.81</td>
<td>0.939</td>
<td>0.061</td>
<td>Yd = 1/(1 + 10^{pK_{dn}-pH})</td>
</tr>
<tr>
<td>0.01</td>
<td>11.31</td>
<td>0.830</td>
<td>0.170</td>
<td>Ya = 1/(1 + 10^{pH-pK_{dn}})</td>
</tr>
<tr>
<td>0.001</td>
<td>10.81</td>
<td>0.608</td>
<td>0.392</td>
<td></td>
</tr>
<tr>
<td>0.0001</td>
<td>10.31</td>
<td>0.329</td>
<td>0.671</td>
<td></td>
</tr>
<tr>
<td>0.00001</td>
<td>9.81</td>
<td>0.134</td>
<td>0.866</td>
<td></td>
</tr>
</tbody>
</table>
C. **pH dependence on the concentration of a WEAK ACID mixed with a STRONG BASE in water**

- Equilibrium dissociation or ionization reaction with NaOH added:
  \[
  [HA] + [Na^+] + [OH^-] \rightleftharpoons [H^+] + [A^-] + [Na^+] + H_2O
  \]
- \( C_a = [HA] + [A^-] \) \quad \( C_B = [Na^+] = \text{total concentration of NaOH added} \)
- \([Na^+] \cong [A^-] \cong C_B \cong Y_d*C_a \)
- \([HA] = C_a - C_B \cong Y_a*C_a \)
- \( pH = pK_{dn} + \log ([A^-]/[HA]) = pK_{dn} + \log (Y_d/Y_a) = pK_{dn} + \log (C_B/(C_a-C_B)) \)
- \( pH = pK_{dn} + \log (C_B/(C_a-C_B)) \)

<table>
<thead>
<tr>
<th>( C_B (M) )</th>
<th>( pH )</th>
<th>( Y_d )</th>
<th>( Y_a )</th>
<th>( pK_{dn} = 5.0 )</th>
<th>( C_A (M) = 1.0 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0001</td>
<td>2.50</td>
<td>0.0001</td>
<td>0.9999</td>
<td>( pH = (1/2)(pK_{dn}-\log C_a) ) if ( C_B &lt;&lt; C_a )</td>
<td>&quot;</td>
</tr>
<tr>
<td>0.001</td>
<td>2.50</td>
<td>0.0010</td>
<td>0.9999</td>
<td>&quot;</td>
<td>&quot;</td>
</tr>
<tr>
<td>0.01</td>
<td>3.00</td>
<td>0.01</td>
<td>0.99</td>
<td>( pH = pK_{dn} + \log (C_B/(C_a-C_B)) )</td>
<td>&quot;</td>
</tr>
<tr>
<td>0.1</td>
<td>4.05</td>
<td>0.1</td>
<td>0.9</td>
<td>&quot;</td>
<td>&quot;</td>
</tr>
<tr>
<td>0.5</td>
<td>5.00</td>
<td>0.5</td>
<td>0.5</td>
<td>&quot;</td>
<td>&quot;</td>
</tr>
<tr>
<td>0.9</td>
<td>5.95</td>
<td>0.9</td>
<td>0.1</td>
<td>&quot;</td>
<td>&quot;</td>
</tr>
<tr>
<td>0.95</td>
<td>6.28</td>
<td>0.95</td>
<td>0.05</td>
<td>&quot;</td>
<td>&quot;</td>
</tr>
</tbody>
</table>

\( Y_d = C_B/C_a \) \quad \( Y_a = (C_a-C_B)/C_a \)

D. **pH dependence on the concentration of a WEAK BASE mixed with a STRONG ACID in water**

- Equilibrium dissociation reaction with HCl added:
  \[
  [CH_3NH_3^+] + [Cl^-] \rightleftharpoons [CH_3NH_2] + [H^+] + [Cl^-]
  \]
- Equilibrium dissociation constant: \( K_{dn} = [H^+][CH_3NH_2]/[CH_3NH_3^+] \)
- \( C_b = [CH_3NH_2] + [CH_3NH_3^+] \) \quad \( C_A = [Cl^-] = \text{total concentration of HCl added} \)
- \( [Cl^-] \cong [CH_3NH_3^+] \cong C_A \cong Y_a*C_b \)
- \( [CH_3NH_2] = C_b - C_A \cong Y_d*C_b \)
- \( pH = pK_{dn} + \log ([CH_3NH_2] / [CH_3NH_3^+]) = pK_{dn} + \log (Y_d/Y_a) = pK_{dn} + \log ((C_b-C_A)/C_A) \)
- \( pH = pK_{dn} + \log ((C_b-C_A)/C_A) \)

<table>
<thead>
<tr>
<th>( C_A (M) )</th>
<th>( pH )</th>
<th>( Y_d )</th>
<th>( Y_a )</th>
<th>( pK_{dn} = 10.62 )</th>
<th>( C_B (M) = 1.0 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0001</td>
<td>12.31</td>
<td>0.980</td>
<td>0.020</td>
<td>( pH = (1/2)(pK_{dn}+\log C_b+14) ) if ( C_A &lt;&lt; C_b )</td>
<td>&quot;</td>
</tr>
<tr>
<td>0.001</td>
<td>12.31</td>
<td>0.980</td>
<td>0.020</td>
<td>&quot;</td>
<td>&quot;</td>
</tr>
<tr>
<td>0.01</td>
<td>12.62</td>
<td>0.99</td>
<td>0.01</td>
<td>( pH = pK_{dn} + \log (Y_d/Y_a) )</td>
<td>&quot;</td>
</tr>
<tr>
<td>0.1</td>
<td>11.57</td>
<td>0.90</td>
<td>0.10</td>
<td>&quot;</td>
<td>&quot;</td>
</tr>
<tr>
<td>0.5</td>
<td>10.62</td>
<td>0.50</td>
<td>0.50</td>
<td>&quot;</td>
<td>&quot;</td>
</tr>
<tr>
<td>0.9</td>
<td>9.67</td>
<td>0.10</td>
<td>0.90</td>
<td>&quot;</td>
<td>&quot;</td>
</tr>
<tr>
<td>0.95</td>
<td>9.34</td>
<td>0.05</td>
<td>0.95</td>
<td>&quot;</td>
<td>&quot;</td>
</tr>
</tbody>
</table>

\( Y_d = (C_b-C_A)/C_b \) \quad \( Y_a = C_A/C_b \)
VI. Understanding buffers

A. Buffers are “molecular sponges”

The term “buffer” is generally used in reference to pH buffers because these regulate pH in the body and are routinely used to control the pH of reaction mixtures in laboratory experiments. However, the buffer concept is really much more general than this and can be applied to any weakly-interacting ligand binding system where a receptor controls the concentration of free ligand by binding or releasing ligand in response to changes in overall ligand concentrations. In other words, buffers “control” or “resist” changes in free ligand concentrations. When H⁺ ions are introduced into a buffered solution, for example, by adding an acid, most of the H⁺ ions will be kinetically adsorbed by unoccupied binding sites of the buffer thereby attenuating the net change in pH. Conversely, when OH⁻ ions are introduced into a buffered solution by adding a base, H⁺ ions kinetically dissociating from the buffer will bind and thereby neutralize free OH⁻ ions to create H₂O and again attenuate the net change in pH. In both cases, the net pH changes observed are not in direct proportion to the amounts of H⁺ or OH⁻ added. In effect, pH buffers are “molecular proton sponges,” binding or releasing protons when the proton or hydroxyl balance shifts. In many cases, pH buffers are simple organic molecules, like acetic acid, that undergo reversible monovalent ligand-binding reactions with protons, as illustrated in Fig. 4.

**FIGURE 4: “Snapshots” of the Acetic Acid/Acetate Equilibrium at Different pH Values**

The “capacity” of acetic acid (HAc) or acetate (Ac⁻) to buffer a solution depends on three conditions: 1) the starting pH of the solution; 2) the pK<sub>dn</sub> of acetic acid; and 3) its total concentration. At pH values below pK<sub>dn</sub> (e.g., pH = 3.7), HAc dominants and the buffer most effectively neutralizes OH⁻ ions added to the solution. At pH values above pK<sub>dn</sub> (e.g., pH = 5.7), Ac⁻ dominants and the buffer most effectively adsorbs added H⁺ ions added to the solution. When pH = pK<sub>dn</sub>, the buffer effectively resists pH changes in both directions. Thus, the *optimal pH* for a buffer is its titration midpoint where 50% of the binding sites are occupied by H⁺ and 50% are unoccupied, on average.

- For a buffer, pK<sub>dn</sub> is measure of its “tendency” to bind or release protons at a given pH.

B. Defining the “buffer range”

The following two questions are typically asked about buffers: What is the “buffer range?” “What is the “buffer capacity?” The *buffer range* for a weak acid or base is the pH range over which the buffer most efficiently neutralizes added H⁺ or OH⁻ ions, as illustrated in Fig.5A and 5B for the titration of acetic acid by either a strong base (top) or strong acid (bottom).
FIGURE 5: “Buffer range” for acetic acid (pKdn = 5.0). (A) [H+] vs. Yd. (B) [H+] vs. Ya.

**NaOH Titration of Acetic Acid: [H+] vs. Yd**

\[ \text{HAc} + \text{Na}^+ + \text{OH}^- \rightleftharpoons \text{Ac}^- + \text{H}_2\text{O} + \text{Na}^+ \]

Dissociation Fraction = \( Y_d = \frac{[\text{Ac}^-]}{[\text{Co}]} \)

\[ [\text{H}^+] = (1-Y_d)^*\text{Kdn}/Y_d \]

pH vs. Yd

\[ \text{pH} = \text{pKdn} - \log((1-Y_d)/Y_d) \]

**HCl Titration of Acetate: [H+] vs. Ya**

\[ \text{Ac}^- + \text{H}^+ + \text{Cl}^- \rightleftharpoons \text{HAc} + \text{Cl}^- \]

Association Fraction = \( Y_a = \frac{[\text{HAc}]}{[\text{Co}]} \)

\[ [\text{H}^+] = Y_a*\text{Kdn}/(1-Y_a) \]

pH vs. Ya

\[ \text{pH} = \text{pKdn} - \log(Y_a/(1-Y_a)) \]

Calculating easy Reversible changes in the H⁺ ion concentration (left y-axis) and pH (right y-axis) with the least amount of change (as defined by the shallowest tangent slope) occurring at the titration midpoint, which defines K_dn or pK_dn as the optimal [H⁺] for pH for buffering effects by this buffer.

- By definition, the buffer range is assumed to extend from pH = pK_dn – 1.0 to pH = pK_dn + 1.0, as bounded by vertical and horizontal hatched lines Figs. 5A and 5B.
- Specifically, the buffer range = pH = pK_dn +/-1.0 for any weak acid or weak base.
- In this pH range, the fractional association, Ya, and the fractional dissociation, Yd, vary between 9% and 91%.
- Specifically, Ya = 9%-91% and Yd = 91%-9% for pH = pK_dn +/-1.0.

Prove these differences with the following relationships: Yd = 1/(1+10\(pK_{dn}-pH\)) and Ya = 1/(1+10\(pK_{dn}-pH\)).

C. Defining the “buffer capacity”

The buffer capacity refers to how much H⁺ or OH⁻ ion a buffer can neutralize at a given concentration and pH. Just as the “distance capacity” of a car is determined by the size of its gas tank, how much gas it can hold, and the miles per gallon it gets, the buffer capacity depends on the total concentration of the buffer (C_o), the total volume of the solution (V_o), the existing pH, and the pK_dn of the buffer. With the expressions for Ya and Yd, it is relatively easy to determine the buffer capacity at any given pH if you know V_o, C_o, and pK_dn.

Example: Consider 100 ml (V_o) of a 0.01 M buffer (C_o) at pH = pK_dn + 0.5. What is the buffer capacity of this buffer? More specifically, how many moles of additional OH⁻ ions could the buffer neutralize by dissociating its bound H⁺ ions assuming equilibrium conditions? Or, how many moles of H⁺ ion could the buffer bind (absorb) under equilibrium conditions with the addition of H⁺ ions?

- The number of moles of H⁺ ion available for neutralizing OH⁻ ions equals total number of moles of HAc at equilibrium:
  \[ HAc \text{ (available)} = [HAc]*V_o = C_o*V_o = C_o*V_o*1/(10^{pH-pK_{dn}+1}) \]
- The number of moles of available H⁺ ion binding sites equals total number of moles of Ac⁻ at equilibrium:
  \[ Ac^- \text{ (available)} = [Ac^-]*V_o = C_o*V_o*Yd = C_o*V_o*1/(10^{pK_{dn}-pH+1}) \]

Calculating the number of moles of H⁺ ion that HAc could release or Ac⁻ could bind at pH = pK_dn + 0.5

\[
HAc = [HAc]*V_o = C_o*V_o*Yd = C_o*V_o*1/(10^{pH-pK_{dn}+1}) \text{ when } C_o = 0.01 \text{ M, } V_o = 0.1 \text{ l and pH = pK_{dn} + 0.5}
\]
\[
= 0.01 \text{ (moles/liter) } * 0.1 \text{ liter } * (1/(10^{pH_{dn}+0.5-pK_{dn}+1}) = 0.0001 * (1/10^{0.5 + 1}) \text{ moles}
\]
\[
= 0.001 * (1/(3.2 + 1)) \text{ moles= 0.0001 * (1/4.2) moles}
\]
\[
= 0.001* (0.24) \text{ moles = 0.00024 moles H⁺ ion available for release and OH⁻ neutralization.}
\]
\[
Ac^- = [Ac^-]*V_o = C_o*V_o*Yd = C_o*V_o*1/(10^{pK_{dn}-pH+1})
\]
\[
= 0.01 \text{ (moles/liter) } * 0.1 \text{ liter} * (1/(10^{pK_{dn}-pK_{dn}+0.5}+1)) = 0.001 * (1/10^{-0.5 + 1}) \text{ moles}
\]
\[
= 0.001 * (10^{0.5}/(1+10^{0.5})) \text{ moles= 0.001 * (3.2/3.2 + 1) moles= 0.001* (3.2/4.2) moles}
\]
\[
= 0.001* (0.76) \text{ moles = 0.00076 moles H⁺ ion that could be bound and removed by acetate ion.}
\]

FIGURE 6: Acid titration of acetic acid (pH = 4.80) at: (A) Ya = 0.25, pH = 5.38, [HAc]/[Ac⁻] = 1:3. (B) Ya = 0.75, pH = 4.32, [HAc]/[Ac⁻] =3:1.
When $Y_a = 3:4 \ (0.75) = [\text{HAc}]/\text{Co} \ (\text{total conc.}), \ \text{pH} = 4.32 \ \text{and} \ [\text{HAc}]/[\text{Ac}^-] = 3:1$. 

http://mcdb-webarchive.mcdb.ucsb.edu/sears/biochemistry/tw-exp/acetate-buffer.htm
D. Visualizing the buffer properties of acetic acid

Reversible ligand binding reactions are dynamic processes, even at equilibrium, because ligand and receptor continuously undergo dissociation and association reactions. At equilibrium, opposite dissociation and association reactions occur at equal rates, so there is no net change in concentration of reactants and products. In the case of acid/base equilibrium reactions, the individual ionized or unionized molecules in solution – i.e., the microstates - continuously bind and release protons while the bulk numbers of ionized or unionized molecules in solution – i.e., the macrostate concentration - remains constant when the solution is at equilibrium. In the case of acetic acid, one microstate is the unionized (protonated) acetic acid (HAc) molecule and the other microstate is the ionized (deprotonated) acetate ion (Ac\(^-\)). The macrostate concentrations - [HAc] and [Ac\(^-\)] - defined by the net concentrations of the individual microstates at equilibrium. As illustrated by Figs. 6A and 6B on the preceding page, the equilibrium macrostate ratios for acetic acid - i.e., \([\text{HAc}]/[\text{Ac}^-]\) – changes with different pH values. When enough strong acid is added to titrate 25% of the acetate ions (Ya = 1/4), [HAc]/[Ac\(^-\)] = 1:3, as shown in the Fig. 6A, and the corresponding [H\(^+\)] concentration increases while the pH decreases to pH = 4.28, about (+) 0.48 pH units above the pK\(_{dn}\), which is assumed to be 4.80. When more strong acid is added to titrate 75% of the acetate ions (Ya = 3/4), [HAc]/[Ac\(^-\)] = 3:1, as shown in the Fig. 6B, and the corresponding [H\(^+\)] concentration increases while the pH decreases to pH = 4.32, about (-) 0.48 pH units below the pK\(_{dn}\). Thus, the majority of protons added to a buffered solution are “sponged up” by available acetate ions as long as these are in excess over the concentration of strong acid added. In this pH range, a 50% increase in fractional saturation (Ya = 0.75 – 0.25 = 0.5) is accompanied by just one unit change in pH (pH = 5.28 – 4.32 = 0.96).

This buffer concept applies to any ligand binding system based on “weak” reversible reactions. Thus, myoglobin and hemoglobin, for example, can also be thought of as “oxygen buffers” because they effectively exhibit the same types of reversible ligand binding properties as weak acids and bases.

E. Biological buffers

Histidine (pK\(_{dn}\) = 6.0) and cysteine (pK\(_{dn}\) = 8.3) are the only two standard amino acids with sidechain pK\(_{dn}\) values near the buffer range needed to maintain physiological pH at or near 7.0. Thus, physiological pH is primarily buffered by other abundant acid/base reaction pairs, such as the phosphate HPO\(_4^{2-}\)/H\(_2\)PO\(_4^{-}\) ion pair (pK\(_{dn}\) = 7.2 at 25\(^\circ\)C) and the HCO\(_3^{-}\)/H\(_2\)CO\(_3\) bicarbonate ion pair (pK\(_{dn}\) = 6.37 at 37\(^\circ\)C), which is particularly important in maintaining blood pH. The effectiveness of the bicarbonate ion pair as a physiological buffer seems surprising since the pK\(_{dn}\) for this acid/base reaction is as least 3.5 pH units below what one expects for a buffer maintaining pH near 7.0. For example, at the blood pH = 7.4, the equilibrium ratio [HCO\(_3^{-}\)]/[H\(_2\)CO\(_3\)] \(\approx\) 7000/1, which would not be expected to result in effective buffering of OH\(^-\) ions. However, aerobically metabolizing organisms produce large steady state amounts of CO\(_2\), which (in humans) dissolves in the blood until exhaled by the lungs. A small fraction (1/300) of the dissolved CO\(_2\) combines with H\(_2\)O to form carbonic acid, H\(_2\)CO\(_3\), a reaction catalyzed by carbonic anhydrase. H\(_2\)CO\(_3\) readily ionizes to the form bicarbonate ion, HCO\(_3^{-}\). Thus, the strong physiological buffering capacity of the bicarbonate ion pair can be explained by large steady state production and reservoir of dissolved CO\(_2\) in rapid equilibration to H\(_2\)CO\(_3\). If the OH\(^-\) ion concentration rises, dissolved CO\(_2\) equilibrates to H\(_2\)CO\(_3\), which in turn equilibrates to HCO\(_3^{-}\) liberating neutralizing H\(^+\) ions. Conversely, if the H\(^+\) ion concentration rises, the protons combine with HCO\(_3^{-}\), which equilibrates to H\(_2\)CO\(_3\) and then dissolved CO\(_2\). Thus, the effective [H\(_2\)CO\(_3\)] concentration in the blood is greater than the actual concentration because dissolved CO\(_2\) acts as a large “buffer” to maintain steady state levels. By taking into account this amount of dissolved CO\(_2\) in blood, as neatly explained by R. Garrett and C. Grisham (Biochemistry, 3\(^{rd}\)
ed. 2005, Thomson, Brooks, and Cole, p. 47), the bicarbonate ion pair has an effective \( pK_{dn} = 6.1 \), which is obviously much closer to physiological pH. Clearly then, anything affecting CO\(_2\) levels in the blood can potentially change blood pH. Hyperventilation (abnormally rapid breathing at rest) for example, reduces dissolved CO\(_2\) (by exhalation) potentially causing respiratory alkalosis (abnormally high blood pH) following the accompanying decrease in \([H_2CO_3]\). Conversely, hypoventilation (abnormally slow breathing at rest) increases dissolved CO\(_2\) levels, (as accumulated through metabolism) potentially causing respiratory acidosis (abnormally low blood pH) following the accompanying increase in \([H_2CO_3]\) and \([H^+]\) and \([HCO_3^-]\).

VII. Environmental perturbation of reversible equilibrium reactions

Not surprisingly, the actual reversible equilibrium properties of any ligand-binding group will depend not only on its chemical structure but also on the nature of its immediate “microenvironment,” including solvent and neighboring atoms in a larger structure. Usually, the \( pK_{dn} \) values listed in textbooks for the ionizable groups of proteins correspond to ionization behavior expected in aqueous environments. However, the ionizable groups in folded proteins may experience localized interactions with neighboring atoms and groups thereby shifting or perturbing their reversible equilibrium properties, sometimes considerably. Such perturbations may or may not have functional significance. In most instances, a perturbed \( pK_{dn} \) value is simply indicative of the fact that a group is positioned near certain kinds of atoms or groups. In some instances, the perturbation may result in function. For example, ionizable groups in enzyme catalytic sites often exhibit altered \( pK_{dn} \) values associated with their participation in catalysis.

A. Differential perturbation of histidine residues in different proteins

The differential ionization behavior for the same chemical group in different microenvironments is illustrated by comparing the acid/base titration behavior of His residues in different proteins by nuclear magnetic resonance (NMR) measurements. With this technique it is possible to monitor the individual ionization reaction of the imidazole ring of a His sidechain. By substituting D\(_2\)O for H\(_2\)O, the magnetic properties of the imidazole ring structure shifts when the deuterium ion binds to unpaired nitrogen electrons of the imidazole ring. Such shifts, measured in “parts per million” (ppm), usually allow one to assign unique \( pK_{dn} \) values for each His residue in a protein.

In the first example shown in Fig. 7 on the next page, quite different \( pK_{dn} \) values are observed for titration of the lone His residues found in either chicken (hen egg-white) lysozyme or human lysozyme (Meadows et al, 1967, PNAS 58: 1307). For chicken lysozyme, the imidazole ring of His 15 titrates with \( pK_{dn} = 5.8 \) while the imidazole ring of His 78 of human lysozyme titrates with \( pK_{dn} = 7.6 \). Considering the 3-D structures of these two enzymes, the dissimilar acid/base titration properties of these two His residues is not really that surprising. As illustrated by the 3-D structures of these two enzymes in Fig. 7, the two His residues are in fact located in substantially different microenvironments, presumably accounting for their observed \( pK_{dn} \) differences. However, both His residues are found well outside the active sites of these enzymes and so the unique ionization properties of these His residues probably has no bearing on actual catalytic functions of the enzymes.
FIGURE 7: Different acid/base titration profiles and structural microenvironments for the lone His residues found in hen egg-white (HEW) lysozyme (His 15, top) and human lysozyme (His 78, bottom).

The lone His 15 imidazole sidechain of hen egg-white lysozyme (HEWL; semi-transparent white surface) displayed here with neighboring atoms.

The lone His 78 imidazole sidechain of human lysozyme (semi-transparent white surface) displayed here with neighboring atoms.

The imidazole ring of histidine sidechains with the unshared electrons of the double bonded N atom having a protonated ring with net charge = +1. The magnetic properties of carbons labeled C2 and C4 are shifted when deuterium binds or dissociates from the ring.

B. Differential perturbation of histidine residues in the same protein

Bovine ribonuclease A (RNase A) has four His residues in - i.e., His 12, His 48, His 105, and His 119 – and as shown in Fig. 8, NMR-monitored titration of the four imidazole rings of these four residues (Meadows et al PNAS 61 408, 1968) shows that each exhibits a unique pKd value consistent with the fact that each is physically situated in a different microenvironment in the folded enzyme as shown in Fig. 9. Interestingly, two of the His residues exhibit additional shifts in their pKd values when the enzyme binds a competitive inhibitor - 3'-cytosine monophosphate (3'-CMP), as also shown in Fig. 8. The assigned pKd value for His 12 undergoes a large shift from 6.2 to 8.0 and the assigned pKd for His 119 undergoes a large shift from 5.8 to 7.4. Presumable, these shifts arise from the interactions between the imidazole sidechains these two His residues and the inhibitor. By contrast, the pKd values assigned to His 48 and His 105 barely shift after inhibitor binds. In summary, these results are consistent with the 3-D structural analysis of RNase A bound to another inhibitor, uridine vanadate, as shown on in Fig. 9, where His 12 and His 119 appear to make direct contact with the inhibitor.

FIGURE 8: Acid/base titration profiles for 4 His residues of bovine ribonuclease A (RNase A) measured by NMR in the absence (closed symbols) or presence (open symbols) of competitive inhibitor, 3'-CMP
C. Two active site histidine residues account for the pH-dependent enzyme activity of RNase A

The large pK_{dn} shifts for **His 12** and **His 119** (but not **His 48** and **His 105**) following competitive inhibitor binding suggest that the **His 12** and **His 119** resides are part of the catalytic site of RNase A and may even participate directly in substrate catalysis through their unique acid/base ionization properties. The *experimentally determined* pH dependent reaction velocity ($V_0$) of RNase A with uridine 2',3'-cyclic phosphate substrate is shown in Fig. 10 (*closed circles*; del Rosario & Hammes, 1969, *Biochemistry* 8:1884). The “bell-shaped” appearance of $V_0$
as a function of pH indicates that enzyme catalysis is directly dependent on the ionization states of at least two ionizable groups, “group 1” and “group 2.” Initially at low pH, $V_0$ rises as pH increases suggesting that group 1 must be deprotonated (ionized) for catalysis. At higher pH, $V_0$ falls off suggesting that when group 2 ionizes enzyme activity drops and the group 2 must therefore be protonated or unionized for effective catalysis to take place. It is proposed that group 1 is His 12 and group 2 is His 119. His 12 is thought to act as a general base in the catalytic reaction in withdrawing a proton from substrate. Thus, His 12 would initially need to be ionized in order to accept a proton. Conversely, His 119 is thought to act as a general acid in the catalytic reaction in donating a proton to a substrate intermediate. Thus, His 119 would initially need to be protonated in order to donate a proton.

This model is reinforced by the experimentally determined pH rate profiles for two measured catalytic constants of this enzyme as shown in Figs. 11A and 11B. The experimentally determined values for, $k'_{cat}$ (Fig. 11A, solid triangles) – i.e., the “apparent” enzyme turnover number, or the number of substrate molecules converted to product per enzyme per unit time - increases with increasing pH or decreasing [H+] until it reaches nearly a constant plateau value at higher pH. In opposite fashion, $K'_{M}$ (Fig. 11B, solid squares) – i.e., the “apparent” Michaelis-Menten constant, or the substrate concentration for half-maximal velocity – is found to be nearly constant at low pH but increases with increasing pH or decreasing [H+]. Increased $k'_{cat}$ values correlate with increased enzyme activity whereas increased $K'_{M}$ values correlate with decreased enzyme activity. With these observations, one can make a simple model for the kinetic properties of RNase A based on the acid/base titration behavior of groups 1 and 2. Assuming that $k'_{cat}$ and $K'_{M}$ both follow the titration profiles for single but different ionizable groups, one can make the following approximations for these two parameters. $k'_{cat} = k_{cat} \cdot Y_{1d}$, assuming $pK_{1dn} = 5.8$, which fairly accurately approximates (open triangles) the observed pH dependent behavior of the observed $k'_{cat}$ (solid triangles) as shown in Fig. 11A. Likewise, $K'_{M} = K_{M}/Y_{2a}$, assuming $pK_{2dn} = 6.2$, which fairly accurately approximates (open squares) the observed pH dependent behavior of the observed $K'_{M}$ (solid squares) as shown in Fig. 11B. When these two pH-dependent constants are combined in the Michaelis-Menten equation for $V_0$, one obtains a theoretical expression for $V_0$ that closely produces a pH-dependent bell-shaped $V_0$ curve (Fig. 10, open circles) that reasonably approximates the experimentally observed curve for $V_0$ (Fig. 10, solid circles). Note that pH = 6.0 is the pH optimum for the enzyme. At this pH, one finds maximum fraction of catalytically active enzyme equals $Y_{1d} \cdot Y_{2a}$ where $pK_{1dn} = 5.8$ and $pK_{2dn} = 6.2$.

In summary, the uniquely perturbed pK_{dn} values for these two histidine residues in RNase A are likely to correspond to His12 as group 1 with $pK_{1dn} = 5.8$ and His119 as group 2 with $pK_{2dn} = 6.2$

VIII. Monovalent O2 binding by myoglobin

Aerobically metabolizing organisms usually trap oxygen from their immediate aqueous or atmospheric environments with specific O2-binding proteins like hemoglobin (Hb), the O2 transport protein of the blood, or myoglobin (Mb), the O2 storage protein of muscle.

Aqueous dissolved O2 is bound by Mb according to the following reversible monovalent equilibrium reactions:

- $\text{Mb(O}_2\text{)} \rightleftharpoons \text{Mb + O}_2 \quad K_{dn} = \frac{[\text{Mb}][\text{O}_2]_{\text{dissolved}}}{[\text{Mb(O}_2\text{)}]}$ dissociation reaction
- $\text{Mb + O}_2 \rightleftharpoons \text{Mb(O}_2\text{)} \quad K_{an} = \frac{[\text{Mb(O}_2\text{)}]}{[\text{Mb}][\text{O}_2]_{\text{dissolved}}}$ association reaction
Michaelis-Menton Equation for $V_o$:

$$V_o = \left[ [E_{tot}] \times k'_{cat} \times [S_o]_o / ([S]_o + K'M) \right]$$

where

- $[E_{tot}]$ equals total enzyme concentration;
- $[S]$ equals initial substrate concentration
- $k'_{cat}$ equals “apparent” turnover number,
- and $K'M$ equals the “apparent” Michaelis-Menton constant

- Theoretical $V_o$:
  
  $$V_o = \left[ [E_{tot}] \times k_{cat} \times Yd1 \times [S]_o / (Km/Ya2) \right]$$

  assuming that $pK1_{dn} = 5.8$, $pK2_{dn} = 6.2$.
- Theoretical $k'_{cat} = k_{cat} \times Yd1$
- Theoretical $K'M = Km/Ya2$
- Theoretical $k'_{cat}/K'M = (k_{cat}/Km) \times Yd1 \times Ya2$

Based on data by del Rosario & Hammes (1969) Biochemistry 8: 1884

http://mcdb-webarchive.mcdb.ucsb.edu/sears/biochemistry/sprdshts/pHprofile-rnase-a.xls

FIGURE 11: pH-dependent behavior of $k'_{cat}$ (left) and $K'M$ (right) values for RNase A.
Measurements of dissolved O₂ concentrations are simplified by Henry’s Gas Law which predicts that

\[ [O₂]_{\text{dissolved}} = \frac{K_{O₂} \cdot p_{O₂}}{1 + \frac{p_{O₂}}{p_{50}}} \]

where \( [O₂]_{\text{dissolved}} \) is the dissolved O₂ concentration, \( K_{O₂} \) is the partition coefficient for O₂ in water in equilibrium contact with a gas containing a given mole fraction of O₂. Thus, the equilibrium equation for O₂ binding to Mb can be rewritten as follows in terms of \( p_{O₂} \) with the equilibrium constant, \( K_{d_n} \), replaced by \( P_{50} \), the partial oxygen gas pressure at which 50% of the Mb molecules have bound O₂ and \( [\text{Mb}] = [\text{MbO₂}] \).

- \( [\text{MbO}_2] \rightleftharpoons [\text{Mb}] + [O₂]_{\text{dissolved}} \quad P_{50} = \frac{[\text{Mb}] \cdot p_{O₂}}{[\text{MbO}_2]} \) \text{ dissociation reaction}
- \( [\text{Mb}] + [O₂]_{\text{dissolved}} \rightleftharpoons [\text{MbO}_2] \quad \frac{1}{P_{50}} = \frac{[\text{MbO}_2]}{[\text{Mb}] \cdot p_{O₂}} \) \text{ association reaction}
- \( \frac{Y_d}{[\text{Mb}] \cdot ([\text{Mb}] + [\text{MbO}_2])} = P_{50} = p_{O₂} / p_{50} = 1 / (1 + 10^{P_{50} - p_{O₂}}) \)
- \( \frac{Y_a}{[\text{MbO}_2] / ([\text{Mb}] \cdot [\text{MbO}_2])} = \frac{p_{O₂}}{P_{50} + p_{50}} = 1 / (1 + 10^{P_{50} - p_{O₂}}) \)

For graphical comparisons between the O₂ saturation of myoglobin and hemoglobin, see Section XI.

IX. **MULTI1VALENT equilibrium ligand binding reactions: Definitions and relationships**

In order to define equilibrium constants for the individual molecular ligand binding steps in a multivalent reaction between receptor and ligand, it is necessary to keep track of each reaction step as the receptor progresses from one physical state, or microstate, to the next. In an association reaction, for example, the reaction step order begins with an empty receptor, with no ligand bound, and ends with a fully saturated receptor having all ligand binding sites occupied. The reverse order of steps holds for the dissociation reaction.

The individual equilibrium constants for each ligand binding site are often referred to as intrinsic or microscopic equilibrium constants because they define the actual physical equilibrium reactions that take place at the molecular level between each individual binding sites and a ligand molecule. Henceforth, intrinsic or microscopic equilibrium association or dissociation constants will be designated with a lower case “\( k_{an} \)” or “\( k_{dn} \)” in order to distinguish them from macroscopic equilibrium constants, which will be designated with an upper case “\( K_{an} \)” or “\( K_{dn} \)” Macroscopic equilibrium constants are generally mass action or bulk thermodynamic equilibrium constants. For example, the association reaction between a bivalent receptor and its ligand might be written as

\( R + 2*L \rightleftharpoons RL_2 \), in which case the equilibrium association constant would be written as follows: \( K_{an} = \frac{[RL_2]}{[R]^2[L]^2} \). In this form, \( K_{an} \) is a mass action equilibrium constant and it does not relate to any single, physically discreet molecular step for ligand binding to one of the two different ligand binding sites of the receptor. Thus, except for monovalent ligand binding reactions, in which only a single molecular ligand binding or dissociation step occurs, macroscopic equilibrium constants are generally different from the microscopic equilibrium constants for the individual reaction steps between ligand and specific ligand binding sites of the receptor.

As discussed below, it is often necessary to keep track of the individual microscopic equilibrium steps in order to correctly describe the ligand bind properties of a multivalent receptor. Because it is intuitively easier to discuss and understand the relative differences between association constants for different reaction steps (as opposed to their relative dissociation constants), most of the reaction schemes discussed in the following sections will be developed in the context of association reactions with intrinsic association constants \( k_{an} \) for the individual reactions steps. However, the intrinsic dissociation constant, \( k_{dn} \), will still come into play when defining the customary \( pk_{dn} \) values for the individual reaction steps.
X. BIVALENT equilibrium ligand binding reactions: Definitions and relationships

A. Bivalent receptors with independent ligand binding sites

Consider a receptor (e.g., protein, macromolecule, enzyme, etc.) - “(L1R2)” - with two distinct binding sites - sites 1 and 2 - for ligand, L (e.g., ion, small molecule, co-receptor, etc.). Next assume that the two sites bind ligand with very different affinities and, thus, have very different dissociation constants. In this case, ligand binding to the receptor will occur in a two-step progression, with the first step association to first binding site (with \( k_{1an} \) being more or less complete (at much lower ligand concentration) before the second step association to the second binding site (with \( k_{2an} \)) starts (at much higher ligand concentration).

Assumptions: \( k_{1an} >> k_{2an} \):

In step 1, ligand associates with site 1 at very low [L] before step 2 where ligand associates with site 2 at relatively high [L]. Thus, ligand has higher affinity for site 1 as compared to its affinity for site 2.

Equilibrium association reactions for a bivalent receptor

- **Step 1** - \( (L1R2) + L \rightleftharpoons (L1R2) \)
- **Step 2** - \( (L1R2) + L \rightleftharpoons (L1R2) \)

**Step 1 equilibrium association constant assuming \( k_{1an} >> k_{2an} \)**

\[
\begin{align*}
\text{Step 1:} & \quad k_{1an} = \frac{[L(L1R2)]}{(L1R2)} = 1/k_{1dn} = 1/[L]_{25} & \text{Empirical definition} \\
& \quad k_{1dn} = [L]_{25}, \text{the ligand concentration at which 25\% of all ligand binding sites are occupied, i.e., with 50\% of site 1 occupied and 0\% of site 2 is occupied.} \\
& \quad pk_{1dn} = -\log k_{1dn} = -\log [L]_{25} \quad \text{constant,} \quad k_{1dn} = 10^{pK_{1dn}} \\
\end{align*}
\]

**Step 2 equilibrium association constant assuming \( k_{1an} >> k_{2an} \)**

\[
\begin{align*}
\text{Step 2:} & \quad k_{2an} = \frac{[L(L1R2)]}{(L1R2)} = 1/k_{2dn} = 1/[L]_{75} & \text{Empirical definition} \\
& \quad k_{2dn} = [L]_{75}, \text{the ligand concentration at which 75\% of all ligand binding sites are occupied, i.e., with 100\% of site 1 occupied and 50\% of site 2 occupied.} \\
& \quad pk_{2dn} = -\log k_{2dn} = -\log [L]_{75} \quad \text{constant,} \quad k_{2dn} = 10^{pK_{2dn}} \\
\end{align*}
\]

Equilibrium fractional association, \( Ya \), assuming that \( k_{1an} >> k_{2an} \):

\[
\begin{align*}
\text{Ya} &= \frac{(2*[(1L_{1}R_{2})] + 1*[(1L_{1}R_{2})] + 0*[(1L_{1}R_{2})]) / 2*[(1L_{1}R_{2})] + [(1L_{1}R_{2})] + [(1L_{1}R_{2})]})}{2*C_{O}} \\
\text{Ya} &= \frac{2*[(1L_{1}R_{2})] + 1*[(1L_{1}R_{2})])}{2*C_{O}} \\
\text{Ya} &= \frac{(0.5* k_{1an}*L + k_{1an}*k_{2an}*L^2) / (1 + k_{1an}*L + k_{1an}*k_{2an}*L^2)}{2*C_{O}} \\
& \text{At 25\% association, } Ya = 0.25, [L]_{25} = k_{1an}. \text{ At 75\% association, } Ya = 0.75,[L]_{75} = k_{2an} \\
\end{align*}
\]

Equilibrium fractional dissociation, \( Yd \), assuming that \( k_{1an} >> k_{2an} \):

\[
\begin{align*}
\text{Yd} &= \frac{(0*[L_{1}R_{2}] + 1*[L_{1}R_{2}] + 2*[L_{1}R_{2}]) / 2*[(L_{1}R_{2})] + [(L_{1}R_{2})] + [(L_{1}R_{2})])}{2*C_{O}} \\
\text{Yd} &= \frac{(1*[L_{1}R_{2}] + 2*[(L_{1}R_{2})])}{2*C_{O}} \\
\text{Yd} &= \frac{(0.5*k_{2dn}*L + L^2) / (k_{1dn}*k_{2dn} + k_{2dn}*L + L^2)}{2*C_{O}} \\
\end{align*}
\]

B. Acid/base titration profile of glycine and other bivalent amino acids

Pure glycine, NH\(_2\)CH\(_2\)COOH, is essentially a chemical “composite” of methyamine and acetic acid (minus one the methyl carbon). When dissolved in water, the weakly acidic carboxylic acid group completely ionizes releasing protons while the weakly basic amino group binds nearly an equivalent number of protons. In effect, the basic group soaks up protons released by the acidic group resulting in a zwitterionic molecule with a negatively-charged...
carboxyl group (-COO\(^{-}\)) and positively-charged amino group almost (-NH\(_{3}\)^{+}). The acid/base titration profile for glycine is shown below with two distinct inflection points corresponding to its two ionizable groups.

**C. Determining the pl (isoelectric pH) of glycine and percentages of 4 equilibrium microstates**

When pure glycine is dissolved in pure water at pH = 7.0 initially, the pH drops a little because the “acid strength” of the carboxylic acid group \(pK_{dn}(COOH) = 2.3\) is 4.7 pH units below neutral pH as compared to the “base strength” of the amino group \(pK_{dn}(NH_{3}^{+}) = 9.6\) which is only 2.6 pH units above neutral pH. The resulting pH change can easily be figured out by applying the condition of electrical neutrality. Biological solutions are always electrically neutral, so that the net concentration of positive charges always equals the net concentration of negative charges. For glycine, this condition results in the following relationship:

- Positive charge concentration = \([H^{+}] + [NH_{3}^{+}]\)
- Negative charge concentration = \([OH^{-}] + [COO^{-}]\)

If the concentration of glycine, \(C_{O} \gg [H^{+}]\) or \([OH^{-}]\) (which are initially equal to 10\(^{-7}\) M), the equation above simplifies to \([NH_{3}^{+}] \approx [COO^{-}]\), or \(C_{O}*[Ya(N)] = C_{O}*[Yd(C)]\). The isoelectric pH = pl is the pH at which the average charge concentration for all ionization states of a molecule equals zero. Thus, the pl is found using the following relationships for \(Ya(N)\) and \(Yd(C)\):

- \(Ya(N) = 1/(1+10^{pl-pK_{dn}(N)}) = 1/(1+10^{pl-pK_{dn}(C)-pl}) = Yd(C)\)

**Solving for pl:**

- \((1+10^{pl-pK_{dn}(C)-pl}) = (1+10^{pl-pK_{dn}(N)})\), and \(10^{pl-pK_{dn}(C)-pl} = 10^{pl-pK_{dn}(N)}\), and
- \(pK_{dn}(C)-pl = pl-pK_{dn}(N)\), and \(2^{*}pl = pK_{dn}(C) + pK_{dn}(N)\), and
- \(pl = (0.5)^{*}(pK_{dn}(C) + pK_{dn}(N))\), the average of the dissociation \(pK_{dn}\) values.

- For glycine, \(pl = (0.5)^{*}(2.3 + 9.6) = 5.9\), which is slightly acidic as predicted.
The fractions or percentages of each of four glycine microstates in an aqueous equilibrium solution can be calculated from the dissociation and fractional associations of the two ionizable groups. These fractions can also be used to calculate the average charge of glycine at any given pH.

<table>
<thead>
<tr>
<th>Unionized or ionized groups</th>
<th>Association/ fractional dissociation</th>
<th>Unionized / ionized group percentages</th>
<th>Glycine microstates in solution</th>
<th>Net charge</th>
<th>Microstate fractions in solution</th>
<th>Microstate percentages in solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>[ NH₃⁺]</td>
<td>Ya(N) = 99.9800514%</td>
<td>[NH₃⁺CH₂COO⁻]</td>
<td>0</td>
<td>Ya(N)*Ya(C) = 99.960107%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>[ NH₂]</td>
<td>Yd(N) = 0.0199486%</td>
<td>[NH₃⁺CH₂COOH]</td>
<td>+1</td>
<td>Ya(N)*Ya(C) = 0.019945%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>[ COOH]</td>
<td>Ya(C) = 0.0199486%</td>
<td>[NH₂CH₂COO⁻]</td>
<td>-1</td>
<td>Yd(N)*Yd(C) = 0.019945%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>[ COO⁻]</td>
<td>Yd(C) = 99.9800514%</td>
<td>[NH₂CH₂COOH]</td>
<td>0</td>
<td>Yd(N)*Ya(C) = 0.000004%</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

D. **Bivalent receptors with identical or very similar ligand binding sites**

Assume that “(1)R₂()” is a “receptor” (e.g., protein, macromolecule, enzyme, etc.) with two identical or similar binding sites, 1 and 2, for ligand, “L” (e.g., ion, small molecule, co-receptor, etc.).

**Equilibrium association reactions for a bivalent receptor with identical or very similar ligand binding sites**

![Diagram](https://via.placeholder.com/150)

For the 1ˢᵗ step of the association reaction, ligand binds to the receptor at either site – i.e., site 1 (top) or site 2 (bottom). For the 2ⁿᵈ step of the association reaction, ligand binds to the receptor at the remaining open site – i.e., site 2 (top) or site 1 (bottom). So far, it is assumed that the association constants for sites 1 and 2 may be different depending on whether the other ligand binding site is already occupied or empty. In other words, the equilibrium constant for L binding to site 1 might differ if site 2 is empty (step 2, top) or occupied (step 2, bottom). In particular, step 1 and step 2 equilibrium constants are likely to be different 1) if the ligand binding sites interact directly with each other; or and 2) if ligand binding at one site indirectly alters the ligand affinity of the second site.

This complex ligand-binding scenario can be simplified if the two binding sites are equivalent and both have exactly the same affinity for ligand binding to the 1ˢᵗ site and both have the same affinity for ligand binding to the 2ⁿᵈ site when one site is already occupied. In this case, it can be assumed that:

- \( k_{1a} = k_{2a} \)
- \( k_{1b} = k_{2b} \)

**Equilibrium fractional association, Ya, assuming that \( k_{1a} \neq k_{2a} \)**
Reversible Ligand Binding Reactions ©Duane W. Sears 9/20/12

- Ya = (2*[(L)1R2(L)]+1*[((L)2R2(L)]+1*[((L)2R2(L)]+0*[((L)1R2(L))] / 2*([(L)1R2(L)]+[(L)1R2(L)]+[(L)1R2(L)])
- Ya = (2*([L]1R2(L)]+1*[([L]2R2(L)]+1*[([L]2R2(L)])) / 2*Co
- Ya = (Ran⁻¹/2*([H⁺]*[H⁺])₅₀ + [H⁺])² / ([H⁺])² + 2*Ra⁻¹/2*([H⁺]*[H⁺])₅₀ + [H⁺])²

- The last equation is found by substituting in the equations for k₁ an and k₂ an and substituting in the following variables: [H⁺]₅₀ = 1/(k₁ an×k₂ an)⁰⁰, which equals the experimentally observed H⁺ ion concentration at 50% saturation, and Ran = k₂ an/k₁ an, which equals the ratio of the equilibrium association constants for 2ⁿ⁻¹ ligand binding step over the 1ˢᵗ ligand binding step.

E. Saturation analysis of length-dependent, anti-cooperative proton binding by di-carboxylates

With curve fitting techniques, the equations above can be used to predict the [H⁺]-dependent fractional saturation as a function of [H⁺] for a bivalent ligand binding receptor. However, have one limitation: they include only one experimentally-determined parameter, [H⁺]₅₀. For a bivalent system, there are two unknowns, — k₂ an and k₁ an — and two independent equations with two independent parameters are required in order to determine two unknowns.

For a monovalent ligand binding system, [H⁺]₅₀ = K dn, but for multivalent systems, [H⁺]₅₀ may or may not correspond to a physical dissociation constant. As discussed in more detail in later sections, [H⁺]₅₀ = K dn for a multivalent receptor only when the receptor binds ligand non-cooperatively. Thus, for non-cooperative bivalent receptor ligand binding, k₂ an = k₁ an, R an = 1, and the equations for Ya and Yd above simplify to the following:

- Ya = [H⁺]/([H⁺] + [H⁺]₅₀) and Yd = [H⁺]₅₀/([H⁺] + [H⁺]₅₀)
- Note that these equations are identical to those describing a monovalent ligand binding receptor.

However, when a bivalent receptor binds ligand either anti-cooperatively or cooperatively, the midpoint of the titration, [H⁺]₅₀ does not correspond to a physical equilibrium constant. It can be shown that [H⁺]₅₀ = (1/k₂ an×k₁ an)¹/² = (k₂ dn×k₁ dn)¹/², sort of an “average” equilibrium constant. In this case, the only way to find exact values for k₂ an and k₁ an with the expressions above is to generate a series of theoretical titration curves for different R an values until one curve superimposes on the observed experimental titration data as shown on the next page. Here the ionization properties of a series of HOOC-(CH₂)ₓ-COOH dicarboxylic acids of varying lengths (“x”) are compared based on pk dn values in R. P. Bell’s, "The Proton in Chemistry," 2nd ed. p. 96, Cornell University Press, Ithica, NY, 1973

Note that the values for R an, pk₁ dn, k₁ an, pk₂ dn, and k₂ dn as indicated on this plot are identified as those describing the titration behavior of succinate (open triangles), which has 2 methylene carbons separating the two carboxyl groups of this di-carboxylic acid. Titration profiles for several other di-carboxylates lead to following patterns emerge.

- A di-carboxylate with only one intervening methylene carbon (malonic acid, “x” = 1) exhibits a saturation curve with the greatest curvature (open squares) as compared to succinate (open triangles). The curve rises sharply as the pH rises from low pH values but “flattens out” as pH values increase to very high values.
- However, the saturation curves for di-carboxylates with greater separation (i.e., with “x” > 2 ) between carboxyl groups show less and less curvature in inverse proportion to “x” and these approach (“x” >> 1) the saturation curve expected for acetic acid with only a single carboxyl group. (line marked with “x” symbols). Nevertheless, the titration curve for adipate, with “x” = 7 (open circles) shows a small amount of curvature relative to the acetic acid titration curve.
F. **Hill analysis of the length-dependent anti-cooperative proton binding by di-carboxylates**

An more rigorous and informative way to find values for $k_{2an}$ and $k_{1an}$ is to generate a **Hill plot** by charting the value for log $(Ya/Yd)$ against log $[H+]$. The resulting line will have a slope at 50% saturation ($S_{50}$), which is mathematically related to $k_{2an}$ and $k_{1an}$. As discussed in several of the following sections, the value of $S_{50}$ on a **Hill plot** is an extremely informative parameter for analyzing multivalent ligand binding reactions.

For a bivalent receptor, the $(Ya/Yd)$ ratio yields the following **Hill equation**:

1. $(Ya/Yd) = (R_{an}^{-1/2}[H^+]^{1/2}[H^+]_{50} + [H^+]^2) / (R_{an}^{-1/2}[H^+]^{1/2}[H^+]_{50} + [H^+]^2_{50})$

On a log-log plot with log $(Ya/Yd)$ recorded along the Y-axis and log $[H+]$ recorded along the X-axis, it can be shown that the slope at 50% saturation for a line on the **Hill plot** for a bivalent receptor will equal:

- $S_{50} = 2*R_{an}^{+1/2}/(R_{an}^{+1/2} + 1)$ at $Ya/Yd = 1$

Using the mathematical relationships for the two **experimentally determined parameters**, $S_{50}$ and $[H^+]_{50}$, if can be shown for a bivalent receptor that in general,

- $k_{1an} = (2 - S_{50})/(S_{50}[H^+]_{50})$; and $k_{2an} = S_{50}/((2 - S_{50})[H^+]_{50})$

**FIGURE 14:** Hill plot analysis – log $(Ya/Yd)$ vs. log $[H+]$ – of the anti-cooperative titration of dicarboxylates - HOOC-(CH2)x-COOH –varying in length (x).
Length-Dependent, Anti-Cooperative Titration Behavior of HCOO-(CH2)x-COOH Di-Carboxylic Acids

<table>
<thead>
<tr>
<th></th>
<th>Succinate, x = 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Malonate, n = 1</td>
<td></td>
</tr>
<tr>
<td>Succinate, n = 2</td>
<td></td>
</tr>
<tr>
<td>Azelate, n = 7</td>
<td></td>
</tr>
<tr>
<td>Dicarboxylate, n &gt;&gt; 1</td>
<td></td>
</tr>
<tr>
<td>Acetic acid</td>
<td></td>
</tr>
<tr>
<td>Ya/Yd (R = 0.200)</td>
<td></td>
</tr>
<tr>
<td>slope (S@50% = 0.618)</td>
<td></td>
</tr>
</tbody>
</table>

H+ ion saturation of succinate: (CH2)2- (COOH)2

The predicted titration parameters are indicated. The line for S_{50} is also shown by the red line.

G. The Hill plot slope at 50% saturation and guidelines for interpreting Hill plots

Several significant conclusions about a bivalent receptor can be made from the value for S_{50}:

1. When S_{50} > 1, R_{an} > 1 and k_{2an}/k_{1an} > 1. By definition, ligand binding is cooperative with the 2nd association constant for binding being greater than the 1st association constant.
2. When S_{50} < 1, R_{an} < 1 and k_{2an}/k_{1an} < 1. By definition, ligand binding is anti-cooperative with the 2nd association constant for binding being less than the 1st association constant.
3. When S_{50} = 1, R_{an} = 1 and k_{2an}/k_{1an} = 1. By definition, ligand binding is non-cooperative with the 2nd association constant for binding being equal to the 1st association constant.
4. S_{50} \leq 2. Specifically, the Hill plot slope 50 saturation can never exceed the valence (n = 2) of a bivalent receptor. In effect, the valence of the receptor is the upper limit for maximum cooperativity for the receptor because the closer S_{50} is to the valence, the more cooperative the reaction.
5. S_{50} > 0. In other words, the Hill plot slope at 50% saturation is always > 0 but never = 0. In effect, the closer S_{50} is to zero, the more anti-cooperative the reaction.
H. Ionization properties of interacting catalytic carboxyl groups in aspartyl proteases

XI. **TRIVALENT equilibrium ligand binding reactions: Definitions and relationships**

A. **Trivalent receptors with distinct ligand binding sites**

Assume that “R₁(1)₂(3)” is a “receptor” (e.g., protein, macromolecule, enzyme, etc.) having 3 distinct binding sites (1, 2, & 3) for ligand, “L” (e.g., ion, small molecule, co-receptor, etc.) that undergo association/association reactions over different ranges of ligand concentration with \( k_{1dn} >> k_{2dn} >> k_{3dn} \)

**Equilibrium dissociation reactions for a trivalent receptor with distinct ligand binding sites assuming \( k_{1dn} >> k_{2dn} >> k_{3dn} \)**

\[
R₁(1)₂(3)L₁ \rightleftharpoons R₁(1)₂(3)L₂ + L \\
R₁(1)₂(3)L₂ \rightleftharpoons R₁(1)₂(3)L₃ + L \\
R₁(1)₂(3)L₃ \rightleftharpoons R₁(1)₂(3)L + L
\]

**Empirical definition**

\[k_{1dn} = 10^{-pk_{1dn}}\]

\[k_{2dn} = 10^{-pk_{2dn}}\]

\[k_{3dn} = 10^{-pk_{3dn}}\]

Fractional association, \( Y_a \), assuming \( k_{1dn} >> k_{2dn} >> k_{3dn} \) or that \( k_{3an} >> k_{2an} >> k_{1an} \)

\[
Y_a = \left[ \left(1.0*\left[R₁(1)₂(3)\right] + 1*\left[R₁(1)₂(3)\right] + 2*\left[R₁(1)₂(3)\right] + 3*\left[R₁(1)₂(3)\right] \right) \right]
\]

\[
Y_a = \frac{3*(k_{1dn}*k_{2dn}*k_{3dn} + k_{1dn}*k_{2dn}*L + k_{1dn}*k_{2dn}*L^2 + k_{1dn}*L^3)}{3*(k_{1dn}*k_{2dn}*k_{3dn} + k_{1dn}*k_{2dn}*L + k_{1dn}*k_{2dn}*L^2 + k_{1dn}*L^3)}
\]

B. **Acid/base titration of histidine and other trivalent amino acids**

Pure histidine, \( \text{NH}_3\text{CH}(-\text{Im})\text{COOH} \), is essentially a chemical “composite” of methylamine, acetic acid, and the imidazole group (R-Im) forming its sidechain R group. When dissolved in pure water (pH = 7), the weakly acidic carboxyl groups completely dissociate while the amino groups binds a somewhat lesser amount of proton. In effect, most of the protons of the carboxyl group “transfer” to the initially unsaturated amino group through reversible ionization reactions, and thus the amino group acts as a buffer “sponging up” free protons. Histidine also has a second albeit much weaker base, the imidazole ring (Im) and a small fraction of this group will also bind protons. Thus, the predominant ionization microstate in solution is expected to be \( \text{NH}_3^+\text{CH}(-\text{Im})\text{COO}^- \), having net charge = 0, along with a small amount of \( \text{NH}_3^+\text{CH}(-\text{ImH}+)\text{COO}^- \) having net charge = +1.

The acid/base titration profiles for histidine (solid line with triangles) and other trivalent amino acids are shown below. Three distinct inflection points are indicative of 3 different ionizable groups where

- \( Y_a = 0.83 \) corresponds to the 50% point for ionization of the COOH group with \( K_{dn}(C) = 1.58 \times 10^{-2} \) and \( pK_{dn}(C) = 2.3 \).
• Ya = 0.5 corresponds to the 50% point for ionization of the imidazole sidechain group Im (K_{dn}(R) = 1. \times 10^{-6} and pK_{dn}(R) = 6.0); and
• Ya = 0.17 corresponds to the 50% point for ionization of the imidazole sidechain group Im (K_{dn}(N) = 6.31 \times 10^{-10} and pK_{dn}(N) = 9.2)

FIGURE 15: Titration of amino acids with three ionizable groups.

http://mcdb-webarchive.mcdb.ucsb.edu/sears/biochemistry/spacingts/trivalent-noninteractive.xls

C. Determining the pI (isoelectric pH) of histidine

When pure histidine is dissolved in pure water (initially, pH = 7.0), the pH will rise a little because two basic groups, the amino group and the imidazole group compete for protons released by the acidic carboxyl group. Just what will the final pH equal? This is easy to figure out remembering that biological solutions are always electrically neutral. That is, the net concentration of positive charges always equals the net concentration of negative charges. For this system, that means:

• net positive charge = [H^+] + [NH_3^+] + [ImH^+] = [OH^-] + [-COO^-] = net negative charge

If the concentration of histidine, C_0, is greater than the initial [H^+] or [OH^-] concentrations (10^{-7}), the equation above simplifies to [NH_3^+] + [ImH^+] \approx [COO^-], or C_0 \times Ya(N) + C_0 \times Ya(R) = C_0 \times Yd(C).

Because the average charge concentration for all ionization states of a molecule must equal zero at this pH, the pH = isoelectric pH (pI). pI is found using the following expressions for Ya(N), Ya(R), and Yd(C):

• Ya(N) + Ya(R) = 1/(1+10^{pI-pKdn(N)}) + 1/(1+10^{pI-pKdn(R)}) = 1/(1+10^{pKdn(C)-pI}) = Yd(C)

Because there is only one negatively-charged acidic group and two partially-charged positive basic groups, the carboxyl group must be fully ionized with negative charge = -1. Thus

• Ya(N) + Ya(R) = 1/(1+10^{pI-pKdn(N)}) + 1/(1+10^{pI-pKdn(R)}) = 1 = Yd(C)

Solving for pI:
• \(1/(1 + 10^{pI-pKdn(N)}) = 1 - 1/(1 + 10^{pI-pKdn(R)}) = (1 + 10^{pI-pKdn(R)} - 1)/(1 + 10^{pI-pKdn(R)})\)
  \(= 10^{pI-pKdn(R)}/(1 + 10^{pI-pKdn(R)}) = 1/(1 + 10^{pKdn(R)-pI})\)

Thus,
• \(1/(1 + 10^{pI-pKdn(N)}) = 1/(1 + 10^{pKdn(R)-pI})\)
• \((1 + 10^{pKdn(R)-pI}) = (1 + 10^{pI-pKdn(N)}); 10^{pKdn(R)-pI} = 10^{pI-pKdn(N)}; \text{ and } pKdn(R)-pI = pI-pKdn(N)\),

Solving for pI
• \(pKdn(R) + pKdn(N) = 2^*pI\)
• \(pI = \frac{1}{2}(pKdn(R) + pKdn(N))\)

\[\text{pI} \approx 0.5^* (6.0 + 9.2) = (0.5)^* (15.2) = 7.6, \text{ the average of the 2 basic group pK}_{dn} \text{ values.}\]

XII. **Cooperative O}_2 **ligand binding by Hemoglobin

The analysis of multivalent ligand binding systems has revealed important nuances concerning the efficiency and regulation of many biological reactions. The classical example is that of hemoglobin (Hb), the oxygen transport protein of blood. The oxygen saturation curves of Hb were found to be “different” than expected by comparisons to simple ligand binding molecules like myoglobin (Mb, see Section VIII above). Namely, Hb binds or releases up to 4 O\(_2\) molecules with a high degree of cooperativity as compared to binding and release of just one O\(_2\) by Mb. Unlike non-cooperative ligand binding systems, the hemoglobin’s association (dissociation) constants for O\(_2\) ligand change in relationship to the number of ligand molecules already bound. For Hb, cooperative ligand binding means that the 1\(^{st}\) O\(_2\) molecule is bound with low affinity while subsequent O\(_2\) molecules are bound with even higher affinity until all Hb binding sites are fully saturated. In the reverse process, the 1\(^{st}\) O\(_2\) molecule is not easily dissociated from saturated Hb, but subsequent O\(_2\) molecules dissociate more easily.

The nature of hemoglobin’s cooperative ligand binding properties was discovered by careful analysis of the shapes of its O\(_2\) saturation curves. The physical mechanism for cooperative ligand binding followed the elucidation of the 3-D structures for two Hb conformations: the deoxy conformation with its four oxygen binding sites empty and having relatively low O\(_2\) affinity; and the oxy conformation with its four oxygen binding sites filled and having relatively high O\(_2\) affinity. Thus cooperative O\(_2\) binding occurs when the low affinity deoxy conformation switches to the high affinity oxy conformation. The overall change in association constant is estimated to be has about a 100-fold. 3-D structure analysis reveals that the O\(_2\) binding sites, i.e., its noncovalently bound heme groups, do not interact directly. Rather, the structure of the whole molecule changes in the presence or absence of bound O\(_2\) resulting in subtle changes in the structures of the heme group, which greatly influence their ability to coordinate with O\(_2\). O\(_2\) binding by Hb is only “half” the process, however, because Hb must also release O\(_2\) in sufficient quantities at suitable locations for energy metabolism. Several metabolic factors also reversibly bind to Hb and fine-tune its net released of O\(_2\) into oxygen-depleted tissues.

A. **Saturation plots for hemoglobin and myoglobin**

The O\(_2\) saturation curves for Hb and Mb are compared in the image on the next page. Clearly, Mb and Hb exhibit very different saturation curves. From the line for Mb saturation (open triangles), it is found that Mb saturates at very low O\(_2\) concentrations compared to Hb; i.e., P\(_{50}\) for Mb is about 2 mm Hg whereas P\(_{50}\) for Hb is about 25 mm Hg. This difference makes perfect “physiological sense” since Mb is an O\(_2\) storage protein that must bind O\(_2\) well, whereas Hb is an O\(_2\) transport protein that must be able to easily bind and deliver O\(_2\) where needed. Thus, in the same physical O\(_2\) environment, bound O\(_2\) on Hb would tend to “flow” to Mb for storage.

• By definition, the fractional saturation for Hb is as follows:
Ya = \(0^*([\text{deoxyHb}] + 1^*[\text{Hb(O}_2\text{)}]_1 + 2^*[\text{Hb(O}_2\text{)}]_2 + 3^*[\text{Hb(O}_2\text{)}]_3 + 4^*[\text{Hb(O}_2\text{)}]_4)\)

\[4^*([\text{deoxyHb}] + [\text{Hb(O}_2\text{)}]_1 + [\text{Hb(O}_2\text{)}]_2 + [\text{Hb(O}_2\text{)}]_3 + [\text{Hb(O}_2\text{)}]_4)\]

- **Note**: Every macrostate concentration with 1, 2, or 3 oxygen molecules bound — i.e., \([\text{Hb(O}_2\text{)}]_1\), \([\text{Hb(O}_2\text{)}]_2\), and \([\text{Hb(O}_2\text{)}]_3\) — represents a composite sum of several microstate concentrations. For example, the macrostate concentration \([\text{Hb(O}_2\text{)}]_1\) is theoretically a composite sum of 4 microstate concentrations each with 1 oxygen bound to 1 of the 4 binding sites of hemoglobin, as indicated below.

- \([\text{Hb(O}_2\text{)}]_1 = [1\text{O}_2] + [1\text{O}_2] + [1\text{O}_2] + [1\text{O}_2]\)

Likewise, \([\text{Hb(O}_2\text{)}]_3\) is also a composite sum of 4 microstate concentrations with 4 ways of binding 3 oxygen molecules to the 4 sites of hemoglobin. The macrostate concentration with 2 bound oxygen molecules - \([\text{Hb(O}_2\text{)}]_2\) — is a composite sum of 6 microstate concentrations. Only \([\text{deoxyHb}]\), with no bound oxygen, and \([\text{oxy-Hb}, [\text{Hb(O}_2\text{)}]_4\] with 4 bound oxygen molecules correspond to 1 microstate.

Because the concentrations of each possible Hb microstate reaction intermediate cannot be accurately measured, the fractional saturation is usually empirically approximated by the “Hill equation” as written below:

- \(Ya = \frac{\text{pO}_2^h}{(\text{pO}_2^h + \text{P}_{50}^h)}\) and \(Yd = \frac{\text{P}_{50}^h}{(\text{pO}_2^h + \text{P}_{50}^h)}\) where \(\text{pO}_2\) = the partial oxygen pressure of air at equilibrium; \(\text{P}_{50}\) = the partial oxygen pressure at 50% oxygen saturation; and \(h\) = the “Hill coefficient,”

- As discussed in the next section, the Hill coefficient equals \(S_{50}\), the slope of the Hill plot line at 50% saturation. For most ligand binding systems the Hill coefficient is determined empirically.

- For Hb in the blood, \(h = 2.8\) as discussed in the next section.

The fact that Hb binds oxygen cooperatively is somewhat evident in the plot of \(Ya\) shown on the next page as indicated by the fact that the saturation curve has a “sigmoid” (“S”-shaped) appearance near the 50% saturation point. As this point saturation rises sharply over a narrow range of \(\text{pO}_2\) values. However, sigmoid shapes can be misleading because even non-cooperative ligand systems will generate sigmoid saturation lines away from the 50% saturation point. A much more definitive analysis of cooperative ligand binding is possible with a Hill plot.

**B. Hill plots for hemoglobin and myoglobin**

With the Hill equation above, it is easy to show that:

- \(\text{Ya/Yd} = \text{Ya}/(1-\text{Ya}) = \left(\text{pO}_2/\text{P}_{50}\right)^h\), or that \(\log(\text{Ya/Yd}) = h (\log \text{pO}_2 - \log \text{P}_{50})\)

As illustrated for Hb and Mb on page 32, a Hill plot records \(\log(\text{Ya/Yd})\) as a function of \(\log \text{pO}_2\). Hill plots for a ligand binding systems that obey the Hill equation exactly will always appear as straight lines with slope = \(h\) (the Hill coefficient) and intersecting the X-axis at 50% saturation where \(\text{Ya/Yd} = 1\) and \(\log(\text{Ya/Yd}) = 0\). By comparison, the Hill plot for Hb saturation is not linear it approaches linearity at the 50% saturation point, the midpoint of the titration. By convention, the slope of Hill plot is taken as the tangent at 50% saturation and this equals the Hill coefficient, \(h\). In the case of Hb, \(h = 2.8\). For Mb, \(h = 1\) and its saturation data produces a straight line throughout the titration.

**FIGURE 16: \(O_2\) Saturation of Hemoglobin, Myoglobin, and Theoretical \(O_2\)-Binding Molecules.**
O₂ Saturation Plots for Hb A & Mb: Observed & Theoretical

- Ya (variable), P₅₀ = 26.5, n = 2.80 (theoretical)
- Myoglobin: P₅₀ = 2, n = 1
- Hemoglobin A in RBCs: P₅₀ = 26.5, n = 2.8
- "100% cooperative" Hb: P₅₀% = 26.5, n = 4 (theoretical)
- "Non-cooperative" Hb: P₅₀% = 26.5, n = 1 (theoretical)
- "Relaxed" OXY-Hb: P₅₀% = 3, n = 1, non-cooperative (theoretical)
- "Tense" DEOXY-Hb: P₅₀% = 252, n = 1, non-cooperative (theoretical)

http://mcdb-webarchive.mcdb.ucsb.edu/sears/biochemistry/spreadsheets/hb-mb-oxygen-saturation.xls
FIGURE 17: Hill Plot for the O₂ Saturation of Hemoglobin, Myoglobin, and Theoretical Molecules.

Hill Plot for Hb A and Mb: Observed & Theoretical

http://mcdb-webarchive.mcdb.ucsb.edu/sears/biochemistry/spreadsheets/hb-mb-oxygen-saturation.xls

C. Significance of the Hill coefficient and guidelines for interpreting Hill plots

There are a number of useful guidelines for interpreting saturation data recorded on Hill plots.

1. Multivalent ligand binding data charted on a Hill plot can produce tangent lines at 50% saturation (S₅₀) with slopes greater than 1 (S₅₀ > 1 or h > 1); or less than 1 (S₅₀ < 1 or h < 1; but S₅₀ > 0 or h > 0); or slope equal to 1 (S₅₀ = 1 or h = 1).

2. When slope = 1 (S₅₀ = 1 or h = 1), the receptor is either monovalent, as shown for Mb (see solid line with triangles having P₅₀ = 2); or it ligand bind non-cooperatively to a multivalent receptor.

3. When S₅₀ < 1 or h < 1; but S₅₀ > 0 or h > 0, it can be shown that ligand binds anti-cooperatively to a multivalent receptor. Namely, initial binding of ligand appears to inhibit or retard subsequent binding of...
more ligand to the same receptor, just as found for the interacting carboxyl groups of di-carboxylates (see Section X).

4. When $P_{50} > 1$, or $h > 1$, it can be shown that ligand binds cooperatively to a multivalent receptor.

   Namely, initial binding of ligand facilitates subsequent binding of additional ligand to the same receptor, just as found for Hb (see solid line with open squares above with X-axis intercept at $P_{50} = 25$).

5. $h_{\text{max}} < n$ where $h_{\text{max}}$ is the maximum possible value for $h$ (i.e., the maximum possible slope of the line on a Hill plot) and $n$ is the receptor valence for ligand (i.e., the receptors total number of ligand binding sites). In effect, the receptor’s valence is the upper limit for maximum cooperativity because the closer $h$ is to $n$ in value, the more cooperative the reaction. However, $h$ can never equal (or exceed) $n$ because this could only happen if the reaction produced no intermediate complexes in reversibly going from its fully desaturated state to its fully saturated state. For example, consider a hypothetical equilibrium interaction between Hb and O$_2$ that produced no intermediate complexes:

   - $\text{deoxyHb} + 4 \text{O}_2 \rightleftharpoons \text{oxyHb(O}_2)^4.$
   - At equilibrium: $P_{50}^4 = [\text{deoxyHb}]^4 \cdot P_{50}^4/[\text{oxyHb(O}_2)^4],$ and $Y_a = 4 [\text{oxyHb(O}_2)^4]/([\text{deoxyHb}] + [\text{oxyHb(O}_2)^4]).$
   - Thus, $Y_a = P_{50}^4/([P_{50}^4 + P_{50}^4]$ and $Y_a/Y_d = (P_{50}/P_{50})^4$
   - The latter equation would yield a straight line on a Hill plot of slope = 4, (see line drawn with long dashes in the preceding figure), this represents a reaction that is not physically possible because intermediate receptor/ligand complexes would have to exist, even if at very low levels.
   - Thus, for Hb with $h = 2.8$ and $h_{\text{max}} = 4 = n$, it is concluded that Hb achieves 70% (i.e., $2.8/4$) of its theoretical maximum degree of cooperative ligand binding.

6. The Hill coefficient gives a minimum estimate of the number of ligand binding sites, or minimum valence $n_{\text{min}}$ of a receptor. Again, in the case of Hb, $h = 2.8$ indicates that $n_{\text{min}} = 3$, since the valence must be an integer (and 2.8 is rounded to 3). To find the exact valence of a multivalent receptor ligand binding data has to be produced and analyzed in a slightly different way. (See discussion of Scatchard analysis in Section XIII).

7. The X-axis intercept for a line on a Hill plot corresponds to the concentration of ligand required for 50% saturation. Thus, Mb achieves 50% saturation at about 13 times lower pO$_2$ ($P_{50} = 2$) than Hb ($P_{50} = 25$) indicating that the O$_2$ affinity of Mb is nearly 13 times higher than the average O$_2$ affinity of Hb.

8. For cooperative ligand binding systems, “high” and “low” ligand binding constants can be estimated from the tangents of slope = 1 drawn from the Hill plot at very high and at very low ligand concentrations as follows:

   - From the tangent drawn at very high pO$_2$ values (tangent of slope = 1 with closed triangles above), $P_{50}^{\text{high}} = 3$, as estimated from the X-axis intercept of this line. This is the approximate 50% saturation point for a theoretical solution of “pure” oxy-Hb that exhibits non-cooperative binding.
   - From the tangent drawn at very low pO$_2$ values (tangent of slope = 1 with closed circles above), $P_{50}^{\text{low}} = 282$, as estimated from the X-axis intercept of this line. This is the approximate 50% saturation point for a theoretical solution of “pure” deoxy-Hb that exhibits non-cooperative binding.
   - Thus, the affinity of Hb for O$_2$ increases nearly a 100-fold as it switches from the low affinity deoxy-Hb conformation ($P_{50}^{\text{low}} = 282$) to the high affinity oxy-Hb conformation ($P_{50}^{\text{high}} = 3$). In other words, Hb’s O$_2$ affinity increases nearly a 100-fold ($282/3$) from conditions of low O$_2$ concentration (e.g., venous blood) to conditions of high O$_2$ concentration (e.g., arterial blood).
D. Biological significance of cooperative ligand binding by hemoglobin

One key aspect of the biological significance of cooperative ligand binding by a receptor is that the receptor can respond more vigorously to fluctuations in ligand concentration, as compared to a comparable receptor that binds ligand non-cooperatively. As shown on the next page, the O2 saturation curves for Hb (solid triangles) under conditions of rest (top figure) or exercise (lower figure) are compared to those expected for a theoretical “non-cooperative” hemoglobin that would bind O2 with a Hill coefficient = 1 throughout non-the saturation process and with the same P50 as Hb A (short dashed line of slope = 1). If one compares the difference between the fractional saturation for Hb in arterial blood (Ya = 98%) as compared to venous blood (Ya = 76%) under conditions of rest, one finds that this difference is 22% as compared to the predicted difference of 19% for “non-cooperative” Hb molecule. Moreover, under conditions of exercise where the PaCO2 drops from 40 mm Hg to 20 mm Hg with increased ventilation (lower figure), the saturation difference equals 67% for as compared to a difference of 37% predicted for “non-cooperative” Hb, in which case the latter would be about 45% less efficient at delivering O2. If Hb were actually this inefficient one would have to a significantly greater body weight in order to achieve the same level of O2 delivery. Assuming the cardiovascular system of average individual weighs about 12 pounds with 5 liters (11 pounds) of blood and 1 pound for the heart and lungs, one’s body would have to increase by need about 28% greater body weight to transport and deliver the same amount of oxygen, or about 3.5 pounds of additional weight. Clearly, the evolution of Hb has streamlined the body weight to some degree.

A second key aspect of the biological significance of cooperative ligand binding by a receptor is that ligand binding process can be more efficiently regulated. In the case of Hb, a number of metabolites or metabolic by-products in the blood regulate the tendency of Hb to bind or O2.

XIII. Determining receptor valence by Scatchard analysis of ligand binding

In order to determine the valence of a receptor, ligand binding data has to determined in such a way that it is possible to establish the exact number of ligand molecules that are bound to receptor, on average, at each equilibrium point measured. Fractional saturation data itself will not provide this information because what is being measured in the fraction of all sites occupied, not the number of sites per receptor. For example, one can carry out a perfectly good saturation spectroscopic analysis of O2 binding by Hb in RBCs, but nothing in the data gathered this way indicates that each Hb molecule can bind up to 4 O2 molecules. If quantitative measures can be made, which provide the equilibrium number of moles of ligand bound per moles of receptor, then the valence of the receptor can be determined. However, it is often difficult to completely saturate the receptor with ligand and so the upper limit to the binding reaction may be ambiguous from direct examination of the data. The method of choice for determining valence from saturation is the Scatchard plot, for two reasons. First, it is easy to extrapolate the data to “infinite” ligand concentration if achieving complete saturation is experimentally limiting. Also, the shapes of lines plotted on Scatchard plots will indicated whether a multivalent receptor binds ligand non-cooperatively, cooperatively, or anti-cooperatively, just like a Hill plot. However, it is more difficult to determine microscopic equilibrium constants from a Scatchard plot (like the 2 constants for a di-carboxylic acid) and the analysis here focuses.

**FIGURE 18: Hill Plot for the O2 Saturation of Hemoglobin, Myoglobin, and Theoretical Models**

<table>
<thead>
<tr>
<th>“Resting” Hb Saturation Curve with PaCO2 = 40 mm Hg</th>
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Reversible-Ligand-Binding-2.doc  RLBR - Page 35 of 42 Pages
Hb Capacity for O₂ Delivery Under Various Environmental and Metabolic Conditions

**FIGURE 19:** Saturation Plot for the O₂ Saturation of Hemoglobin as a Function of Altitude (top) and Body Temperature (bottom)

http://mcdb-webarchive.mcdb.ucsb.edu/sears/biochemistry/spreadsheets/hb-mb-oxygen-saturation.xls
Environmental and Metabolic Affects on O₂ Delivery by Hb A

- Hb A at 14,862 ft (Lima Peru), EXCERSIZING AFTER ACCLIMATION
- Hb A at TEMPERATURE = 40 oC (FEVER)

http://mcdb-webarchive.mcdb.ucsb.edu/sears/biochemistry/sprdshts/hb-multiregulation.xls
FIGURE 20: Saturation Plot for the $O_2$ Saturation of Hemoglobin as a Function of Blood pH (top) and Carbon Monoxide (bottom)

Environmental and Metabolic Affects on $O_2$ Delivery by Hb A

Hb A at pH = 7.2 in VENOUS BLOOD RESTING AT SEA LEVEL

Environmental and Metabolic Affects on $O_2$ Delivery by Hb A

ACUTE CARBON MONOXIDE POISONING (25% CO)

http://mcdb-webarchive.mcdb.ucsb.edu/sears/biochemistry/sprdshts/hb-multiregulation.xls
on using Scatchard plots to establish valence.

In order to determine the valence of a receptor, ligand binding data has to determined in such a way that it is possible to establish the exact number of ligand molecules that are bound to receptor, on average, at each equilibrium point measured. Fractional saturation data itself will not provide this information because what is being measured in the fraction of all sites occupied, not the number of sites per receptor. For example, one can carry out a perfectly good saturation spectroscopic analysis of O₂ binding by Hb in RBCs, but nothing in the data gathered this way indicates that each Hb molecule can bind up to 4 O₂ molecules. If quantitative measures can be made, which provide the equilibrium number of moles of ligand bound per moles of receptor, then the valence of the receptor can be determined. However, it is often difficult to completely saturate the receptor with ligand and so the upper limit to the binding reaction may be ambiguous from direct examination of the data. The method of choice for determining valence from saturation is the Scatchard plot, for two reasons. First, it is easy to the extrapolate the data to “infinite” ligand concentration if achieving complete saturation is experimentally limiting. Also, the shapes of lines plotted on Scatchard plots will indicated whether a multivalent receptor binds ligand non-cooperatively, cooperatively, or anti-cooperatively, just like a Hill plot. However, it is more difficult to determine microscopic equilibrium constants from a Scatchard plot (like the 2 constants for a di-carboxylic acid) and the analysis here focuses on using Scatchard plots to establish valence.

The Scatchard plot is based on the Scatchard equation, which is easy to derive from the fractional saturation equation. At equilibrium, the average number of ligand molecules bound per receptor molecule (r) just equals the valence (n) times the fractional saturation (Ya). (Note: discussions of the Scatchard equation usually use the symbol, “n” for valence so as not to confuse it with the Hill coefficient, h.)

Thus, for a monovalent receptor at equilibrium:

- \[ r = n \times Ya = n \times [L] / ([L] + K_{dn}) \]
  Rearranging,
- \[ r \times [L] + r \times K_{dn} = n \times [L] \]
- \[ n \times [L] - r \times [L] = (n - r) \times [L] = r \times K_{dn} \]
  Finally,
- \[ \frac{r}{[L]} = \frac{n - r}{K_{dn}} \]
  Scatchard equation

Thus, by plotting \( r/[L] \) vs. \( r \), (a Scatchard plot) one should observe a straight line with slope \( -K_{dn} \) or \( -1/K_{dn} \) and an X-axis intercept \( = v \) when \( [L] >> 0 \) and \( r/[L] \) approaches zero. This behavior is found for Mb (closed triangles) in the top plot below and acetic acid (“x” symbols) in the bottom plot below.

For multivalent systems, one still obtains an equation for \( r \) using \( n \times Ya \) because this is the average number of ligand molecules bound at equilibrium. If ligand binding is non-cooperative, the Scatchard will still yield a straight line of slope \( -1/K_{dn} \) because only one equilibrium constant describes the system. However, if ligand binding is cooperative or anti-cooperative, the line on a Scatchard plot will deviate predictably form linearity as illustrated by the examples on the previous page. Note that the cooperative binding of Hb produces a full arc on a Scatchard plot where the anti-cooperative binding of the di-carboxylates produces a “boomerang” effect which is more pronounced with greater cooperativity (compare malate with azelate, for example. In all cases, the X-axis intercepts equal the valence of the receptor extrapolate to the valence of the respective receptors.
Reversible Ligand Binding Reactions

FIGURE 21: Scatchard Plots for the O₂ Saturation of Hemoglobin and Myoglobin (top) and Dicarboxylates of Varying Lengths (bottom)

Scatchard Plots for Hb A & Mb:

Observed & Theoretical

<table>
<thead>
<tr>
<th>r/pO₂ = v*Ya/pO₂ mm Hg⁻¹</th>
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<tbody>
<tr>
<td>r/v*Ya = mole bound ligand/mole of Hb A or Mb</td>
</tr>
</tbody>
</table>

Hemoglobin A in RBCs: P50 = 26.5, n = 2.8
Ya (variable), P50 = 26.5, n = 2.80 (theoretical)
Myoglobin: P50 = 2, n = 1
"100% cooperative" Hb: P50% = 26.5, n = 4 (theoretical)
"Non-cooperative" Hb: P50% = 26.5, n = 1 (theoretical)
"Relaxed" OXY-Hb: P50% = 3, n = 1, non-cooperative (theoretical)
"Tense" DEXOXY-Hb: P50% = 252, n = 1, non-cooperative (theoretical)
Linear (Myoglobin: P50 = 2, n = 1)

Length-Dependent, Anti-Cooperative Scatchard Plot

of HCOO-(CH₂)ₓ-COOH Di-Carboxylic Acids

Succinate, x = 2

<table>
<thead>
<tr>
<th>Dixon–Pertl n-x plots of HCOO-(CH₂)ₓ-COOH Di-Carboxylic Acids</th>
</tr>
</thead>
<tbody>
<tr>
<td>v*Ya/[H+] = r/[H+]</td>
</tr>
</tbody>
</table>

Malonate, n = 1
Succinate, n = 2
Azelate, n = 7
Dicarboxylate, n >> 1
Acetic acid
Ya (R = 0.200)

0 < Ran < 1, Anti-Cooperative
pkdn1 = 5.05
Ran = 0.200
pkde2 = 4.35

http://mcdb-webarchive.ucsb.edu/sears/biochemistry/sprdshts/hb-mb-oxygen-saturation.xls
http://mcdb-webarchive.ucsb.edu/sears/biochemistry/sprdshts/bivalent-interactive.xls
XIV. **Cooperative and anti-cooperative mechanisms of enzyme regulation**

A. **Bacterial aspartate transcarbamoylase (ATCase)**

Hill plot analysis of the reactions catalyzed by complex multi-subunit enzymes follows from rearrangement of the Michaelis-Menten equation to define a “fractional velocity” parameter, $V_O/V_{max} = [S_O]/([S_O] + K_M)$ where $V_{max} = k_{cat}*[E_{tot}]$. This parameter is akin to Hill’s fractional saturation, $Y_a$, and, as such, it also leads to the following definition for a $Y_d$-like parameter, $(V_O-V_{max})/V_{max} = K_M/([S_O] + K_M)$. In accordance with Eqs. (5) and (6) above, a general Hill equation can be written for a multivalent enzyme catalyzed reaction as follows:

- $V_O/(V_{max} - V_O) = ([S_O]/K_M)^h$
- $\log (V_O/(V_{max} - V_O)) = h*\log ([S_O]/K_M)$

As before, the Hill coefficient, $h$, is a measure of the anti-cooperative, cooperative, or non-cooperative catalytic response of a complex enzyme to the initial substrate concentration, $[S_O]$.

In this way, it is possible to characterize by Hill plot analysis, the allosteric conformational transitions that regulate the kinetics of some enzymes like aspartate transcarbamoylase (ATCase). ATCase is dodecameric enzyme complex consisting of 6 identical catalytic subunits and 6 identical regulatory subunits as shown by X-ray diffraction studies (H. L. Monaco, et al., 1978, *Proc Natl Acad Sci U S A.*, 75:5276) and Scatchard analysis (J. O. Newell, et al, 1989, *J Biol Chem. 264*:2476). When published catalytic data (J. C. Gerhart and A. B. Pardee, 1962, *J Biol Chem. 237*: 891) for this enzyme is re-plotted according to a Hill plot, as illustrated in Fig. 22 on the next page, one finds that catalysis by ATCase is cooperative ($h = 2.0$ to $2.8$) with respect to the initial aspartate concentration, $[\text{Asp}]_0$, one of its two substrates for this reaction. As also shown in this figure, ATP and CTP (two metabolites that bind to the regulatory subunits of ATCase and regulate its activity in bacteria) significantly affect the activity of the enzyme by shifting its apparent $K_M$ at $V_O/(V_{max} - V_O) = 1.0$. Whereas $2.0$ mM ATP enhances enzyme activity by shifting the titration curve to the left, $0.4$ mM CTP inhibits enzyme activity by shifting the titration curve to the right. Finally, after treating ATCase with $1$ M HgNO$_3$ (a sulfhydryl alkylating agent), it loses its cooperative catalytic properties and reverts to an enzyme with non-cooperative catalytic properties ($h = 1.0$), as also shown in Fig. 5. Because this agent partially disassembles ATCase into smaller subunit complexes (J. C. Gerhart and A. B. Pardee, 1962, *J Biol Chem. 237*: 891), the cooperative catalytic properties of this enzyme appear to stem from the native dodecameric structure of this enzyme.
FIGURE 22: Hill plot analysis of ATCase enzyme activity in terms of the initial rate of \( N \)-carbamoyl aspartate production as function of the initial aspartate concentration, \([\text{Asp}])_0\), in the presence of an excess carbamoyl phosphate, the other substrate in this reaction.

The Hill coefficient, \( h \), for each data set was determined by approximating the slopes of the lines connecting data points at \([\text{Asp}])_0\) yielding 50% \( V_{\text{max}} \).


http://mcdb-webarchive.mcdb.ucsb.edu/sears/biochemistry/sprdshts/atcase.xls