COOPERATIVITY, ALLOSTERY, SYMMETRY, AND LINKAGE.

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Abstract

Cooperative ligand binding is a fundamental function of a large number of proteins, whose physiological relevance spans from transport, catalysis and regulation of the cell cycle. The reversible oxygen combination to hemoglobin is a prototype of cooperativity and has been convincingly demonstrated to be a consequence of allostery, the ability of the protein to adopt either of (at least) two structural and energy states. The simplest theoretical framework that correlates cooperativity and allostery is the two-state model originally proposed by Monod, Wyman and Changeux (MWC) in 1965, whose critical assumption is that of perfect structural symmetry.

This lecture is meant to provide a general intrduction to some crucial properties of, and concepts relating to, cooperativity and allostery, namely:

1) a taxonomy of chemical linkage types
2) heterotropic linkage
3) homotropic linkage
4) cooperativity as a special case of linkage
5) homotropic and heterotropic linkage in hemoglobin

6) the two-state model of cooperativity and some of its alternatives.

1. The discovery of homotropic and heterotropic linkage.

In 1904 Bohr, Hasselbalch and Krogh published, in the same paper, two momentous biochemical discoveries, under the title *Concerning a Biologically Important Relationship - The Influence of the Carbon Dioxide Content of Blood on its Oxygen Binding*. They found that:

(i) the O_2 affinity of hemoglobin (Hb) increases as the gas concentration (or partial pressure) increases; i.e. *saturation is a sigmoidal, rather than hyperbolical, function of P_{O2}.* This effect was, and still is, called cooperativity. Cooperative O_2 binding by Hb was the first member of a large class of events that we call ***homotropic linkage***.

(ii) The O_2 affinity of Hb decreases as the hydrogen ion and carbon dioxide concentrations increase. The effect of pH is appropriately called the Bohr effect. These phenomenona were the first instances of the large class of events that we call ***heterotropic linkage***.

Bohr's original data:

Curve labels: Pferdeblut 5 mm CO_2, 5, 10, 20, 40, 80 mm CO_2
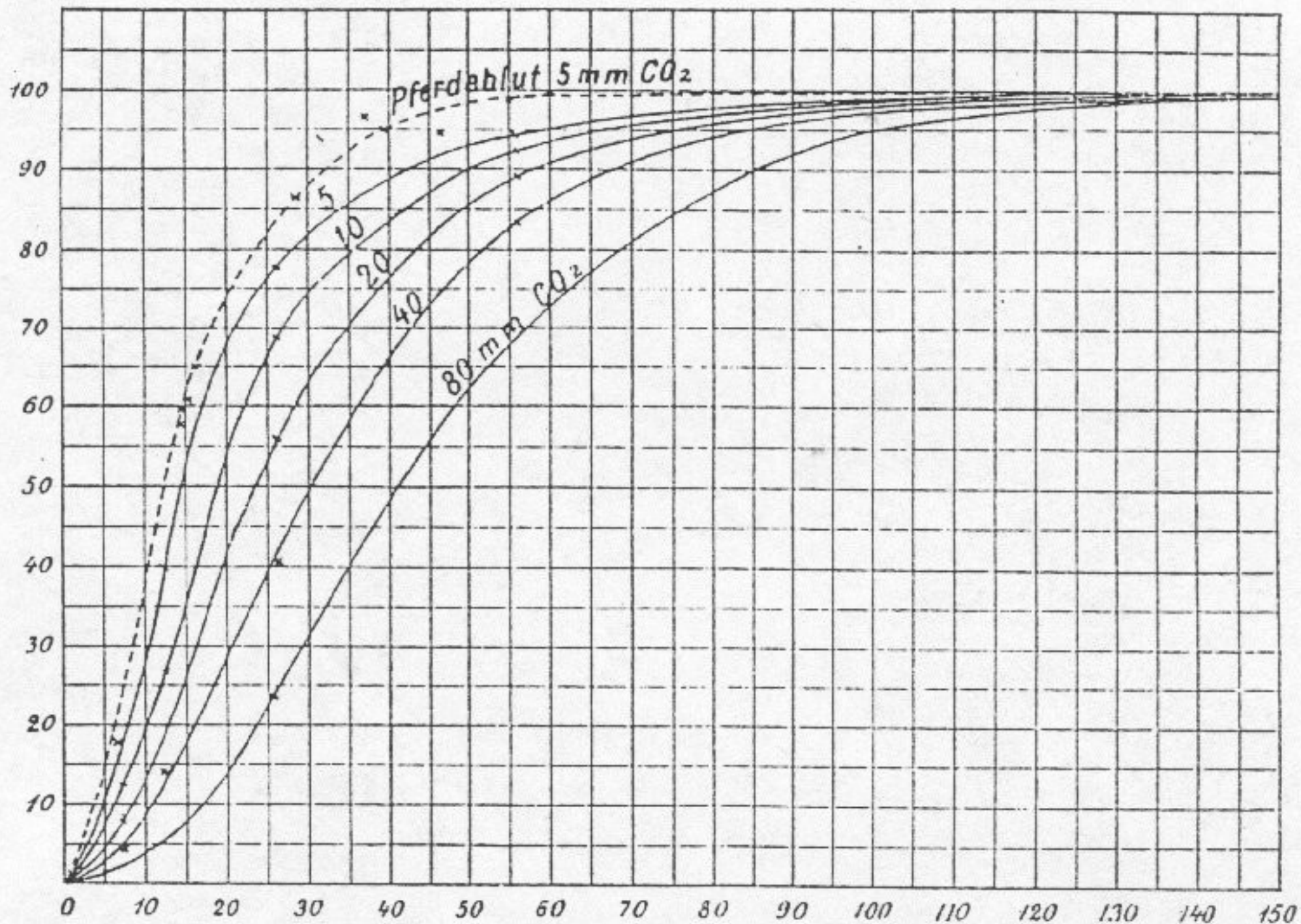
2. A general definition of chemical linkage.

In modern terms, Bohr had found two tipes of **chemical linkage**.

Linkage occurs whenever a (biological) macromolecule binds two or more ligands to the same, or to equivalent, or to different sites; and each ligand influences the binding affinity of the other(s).

Thermodynamics dictates that if ligand #1 influences the affinity of the macromolecule for ligand #2, then the opposite is also true, and ligand #2 affects the affinity for ligand #1, in the same direction. This point (and many others) were demonstrated by Jeffries Wyman in 1948, and in an extended form, in 1964.

For the purposes of this lecture, we adopt the definitions by Wyman and Gill, i.e.:

(i) if two different ligands compete for the same binding site of the macromolecule, we have a case of ***identical linkage***: e.g. O_2 and CO competing for the heme iron of myoglobin (Mb).

(ii) if two (or more) molecules of the same ligand bind to two (or more) equivalent binding sites of the same macromolecule, we have a case of ***homotropic linkage***: e.g. O_2 and Hb,Hb being a tetramer and binding four molecules of O_2. If the ligand affinity increases as more ligand is bound we have positive homotropic linkage (i.e. cooperativity); otherwise we have negative homotropic linkage.
Take home message #1: homotropic linkage requires more than one binding site for the same ligand.

(iii) if two (or more) different ligands bind to two (or more) non equivalent binding sites we have ***heterotropic linkage*** (positive if each ligand increases the affinity for the other, negative otherwise): e.g. the Bohr effect or the effect of DPG on the O_2 affinity of Hb.

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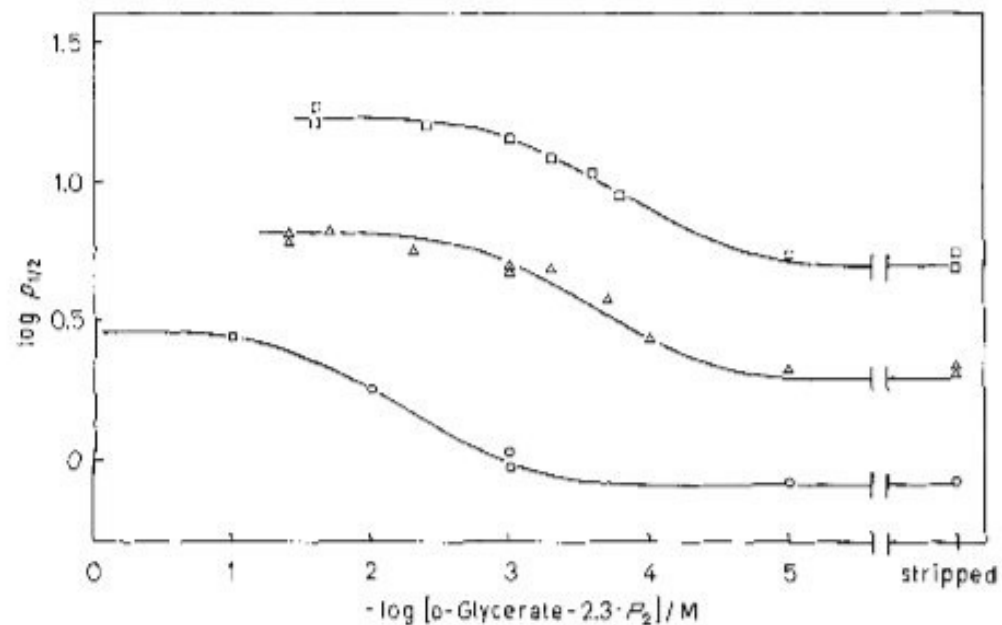
Fig. 1. *Effect of D-glycerate-2,3-P₂ concentration on the oxygen affinity of normal human hemoglobin at different pH values.* (□) pH 6.5, (△) pH 7.5, (○) pH 8.6. 0.1 M Bistris or Tris/HCl + 0.1 M NaCl at 20 C. [Hb] ≈ 60 μM tetramer
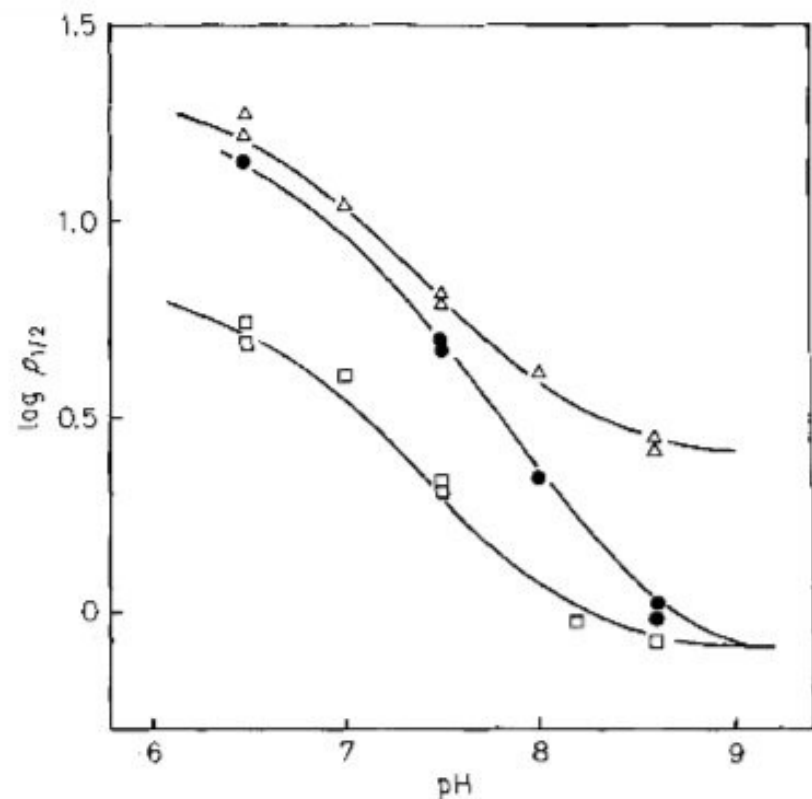
Fig. 2. *Oxygen Bohr effect of normal human hemoglobin at 20 °C.* (□) Stripped HbA, (●) in presence of 1 mM D-glycerate-2,3-P₂, (△) in presence of saturating amount of D-glycerate-2,3-P₂. 0.1 M Bistris or Tris/HCl buffer + 0.1 M NaCl. [Hb] ≈ 60 μM tetramer

3. The physical chemistry of heterotropic linkage with 1:1 stoichiometry.

The thermodynamics of heterotropic linkage was described in quantitative terms by Jeffries Wyman in 1948.

The chemical reaction scheme for a protein (P) binding two different ligands (L and M) to two different sites is as follows:
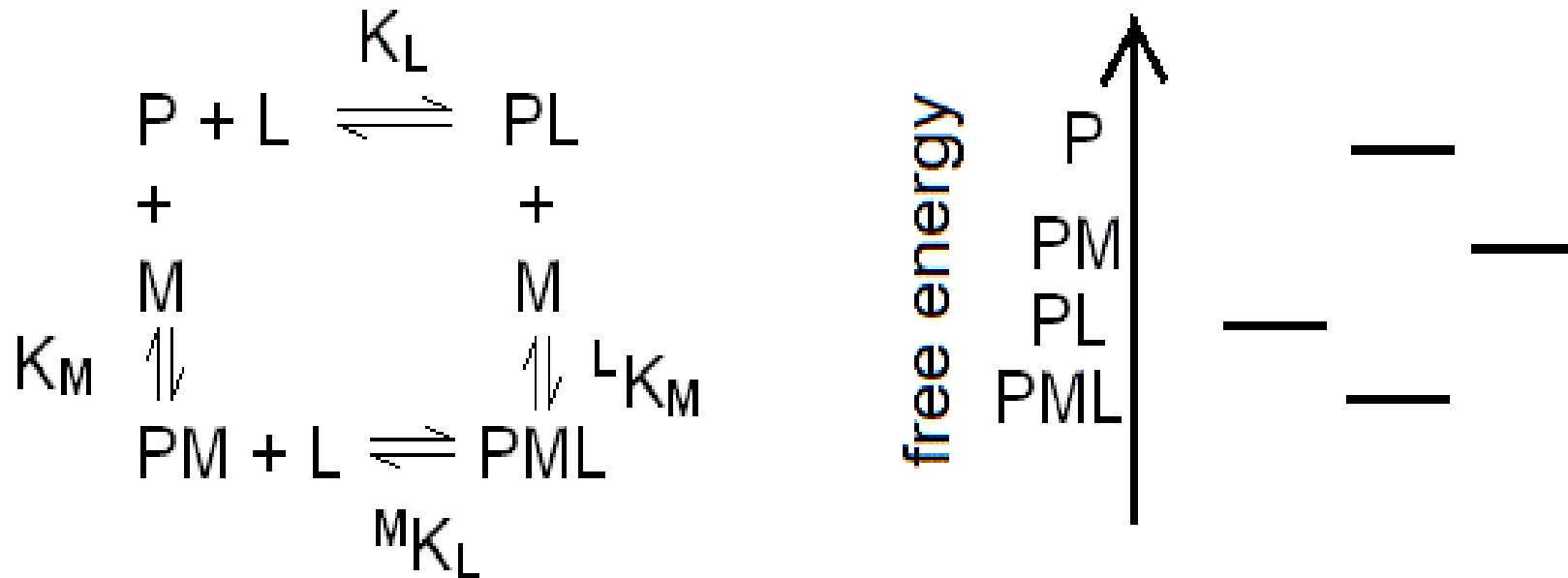
The pertinent equilibrium constants are defined as:

$K_L = [P][L] / [PL]$ (eqn. 3a)
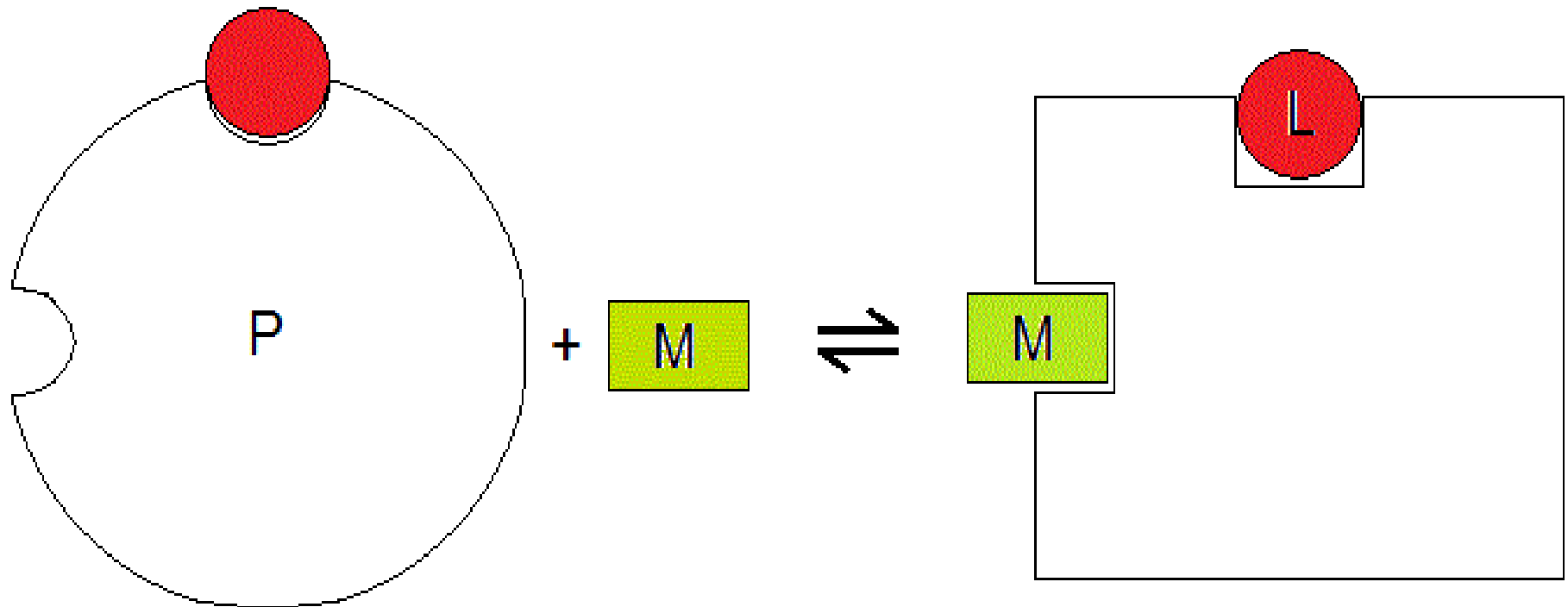
$K_M = [P][M] / [PM]$ (eqn. 3b)

$^{M}K_L = [PM][L] / [PML]$ (eqn. 3c)

$^{L}K_M = [PL][M] / [PML]$ (eqn. 3d)

The linkage is defined **positive** if $^MK_L<K_L$ (ligand M increases the affinity of the protein for ligand L), **negative** otherwise.

Thermodynamics requires that if $^MK_L<K_L$ then $^LK_M<K_M$, and *vice-versa*.

We can visually imagine a case of negative heterotropic linkage as in the cartoon below:

Four states of P are populated in our system: P, PM, PL, PML; we can chose any of them as the reference species and express all concentrations as a function of the concentration of the reference species. We take P as the reference species and we obtain:

$$[PL] = [P]\,[L] / K_L \qquad\qquad\qquad\qquad\qquad (eqn.\ 3e)$$

$$[PM] = [P]\,[M] / K_M \qquad\qquad\qquad\qquad\qquad (eqn.\ 3f)$$

$$[PML] = [PM]\,[L] / {}^{M}K_L = [P]\,[M]\,[L] / (K_M\ {}^{M}K_L) \qquad (eqn.\ 3g)$$

$$[PML] = [PL]\,[M] / {}^{L}K_M = [P]\,[M]\,[L] / (K_L\ {}_{L}K_M) \qquad (eqn.\ 3h)$$

The last two equations yield:

$$K_M\ {}^{M}K_L = K_L\ {}^{L}K_M \qquad or \qquad {}^{L}K_M = K_M\ {}^{M}K_L / K_L \qquad (eqn.\ 3i)$$

Take home message #2: *as a consequence of energy conservation, one of the equilibrium constants of a thermodynamic square can be derived from the other three.*

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$$[PL] = [P] [L] / K_L \qquad\qquad\qquad\qquad\qquad \text{(eqn. 3e)}$$

$$[PM] = [P] [M] / K_M \qquad\qquad\qquad\qquad\qquad \text{(eqn. 3f)}$$

$$[PML] = [PM] [L] / {}^{M}K_L = [P] [M] [L] / (K_M {}^{M}K_L) \qquad \text{(eqn. 3g)}$$

$$[PML] = [PL] [M] / {}^{L}K_M = [P] [M] [L] / (K_L {}_{L}K_M) \qquad \text{(eqn. 3h)}$$

The last two equations yield:

$$K_M {}^{M}K_L = K_L {}^{L}K_M \qquad \text{or} \qquad {}^{L}K_M = K_M {}^{M}K_L / K_L \qquad \text{(eqn. 3i)}$$

Take home message #2: *as a consequence of energy conservation, one of the equilibrium constants of a thermodynamic square can be derived from the other three.*

The **binding polynomial** of P is the sum of the concentrations of all species of P, expressed as functions of the reference species:

$$[P_{tot}] = [P] [(1 + [L] / K_L) + [M]/K_M (1 + [L] / {}^M K_L)] \qquad \text{(eqn. 3j)}$$

It is important to keep track of the chemical meaning of the terms of the binding polynomial: the first represents [P], the second [PL], the third [PM] and the fourth [PML].

The fractional saturation of P with L results:

$$Y_L = ([PL] + [PML]) / ([P] + [PL] + [PM] + [PML]) =$$

$$= \frac{[L]/K_L + [M][L]/K_M {}^M K_L}{(1 + [L]/K_L) + [M]/K_M (1 + [L]/ {}^M K_L)} \qquad \text{(eqn. 3k)}$$

We remark that:

(i) if $^MK_L = K_L$, the presence of M has no effect on the binding of L, and our equation reduces to:

$$Y_L = [L] / (K_L + [L])$$

(ii) if [M] = 0 (and, of course, [PM] = 0 and [PML] = 0) our equation reduces to:

$$Y_L = [PL] / ([P] + [PL]) = [L] / (K_L + [L])$$

(iii) if [M] >> K_M and [M] >> $^LK_M = K_M \, ^MK_L/K_L$
(this condition implies that only protein species containing M are populated, i.e. [P] = 0 and [PL] = 0), our equation reduces to:

$$Y_L = [PML] / ([PM] + [PML]) = [L] / (^MK_L + [L])$$

If none of the above considered simplifications apply, we should use the full equation for Y_L and we obtain a value of $K_{L\ app}$ which is intermediate between K_L and ^MK_L.

The experiment is usually carried out at fixed concentration of M and variable concentration of L, and one can rewrite for simplicity eqn. 3k using the following terms:

$$\alpha = {}^MK_L\ K_M + [M]\ K_L$$

$$\beta = K_L\ {}^MK_L\ (K_M + [M])$$

$$Y_L = [L]\ \alpha\ /\ (\beta + [L]\ \alpha) = [L]\ /\ (\beta/\alpha + [L]) \qquad \text{(eqn. 3k')}$$

Eqn. 3k' describes a hyperbola, and is identical to a simple binding equation, except that the apparent dissociation constant, β/α, is a complex function of three constants (K_L, ^MK_L, and K_M) and one variable ([M], which however is kept constant during each single determination).

Heterotropic linkage in a monomeric protein: ligand M changes the affinity of the protein for ligand L, but does not cause cooperativity. Ligand binding isotherms are hyperbolic, and simply right (or left) shifted. The $L_{1/2}$ is a hyperbolic function of [M].

Heterotropic effects in a monomeric protein

$$P + L \rightleftharpoons PL$$
$$+M \qquad\qquad +M$$
$$PM + L \rightleftharpoons PML$$

$${}^{M}K_L = 10\, K_L$$
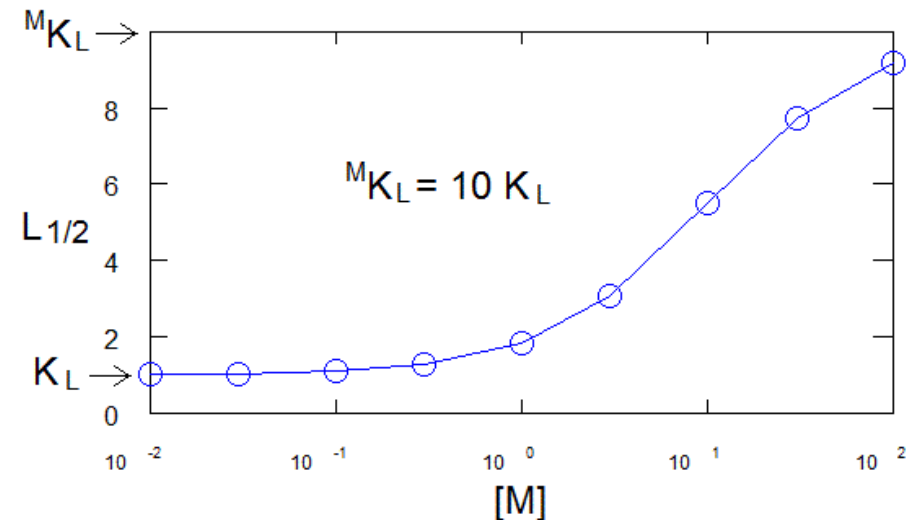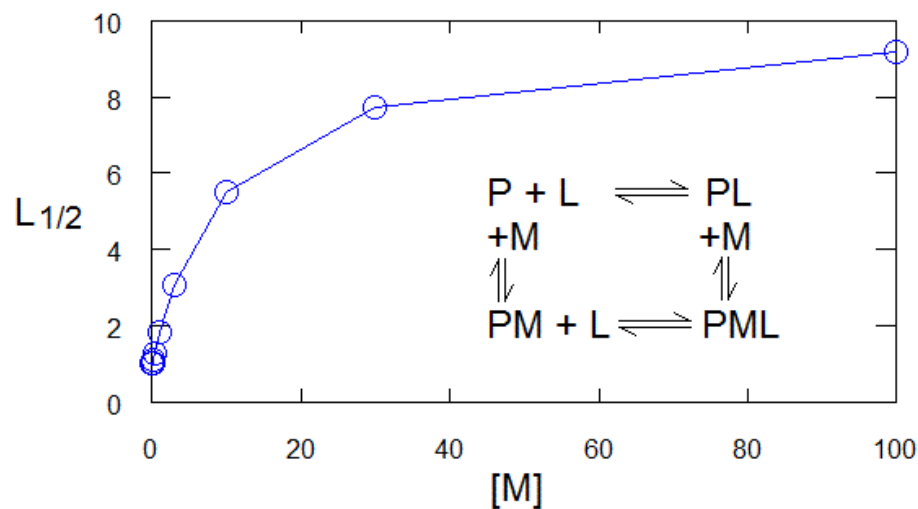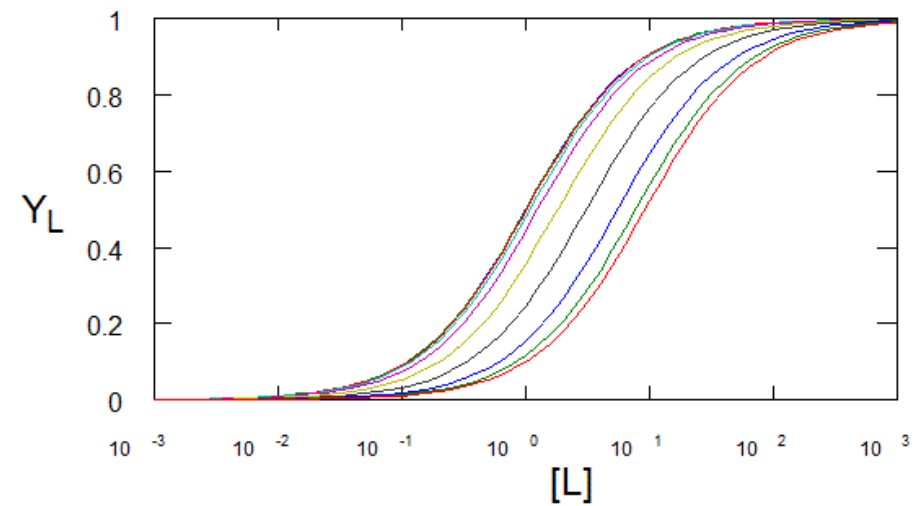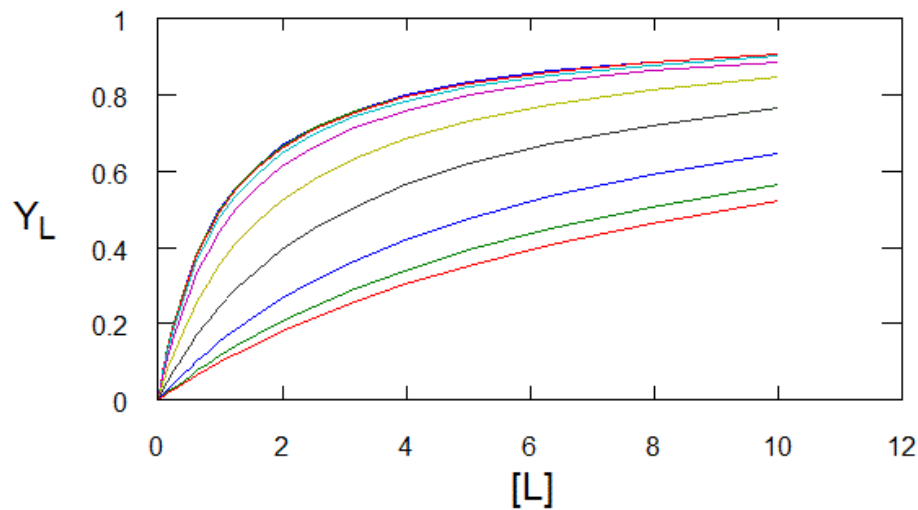
4. Ligand binding to the non-cooperative homodimer

The thermodynamic properties of the oligomer will be described for the simplest possible case, that of a symmetric homodimer

$$P + 2\,L \rightleftharpoons PL + L \rightleftharpoons PL_2 \qquad \text{(eqn. 4a)}$$
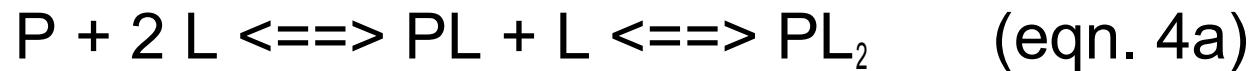
In this system each subunit binds one molecule of the ligand and is insensitive to what happens to its partner in the dimer, hence the binding polynomial and the fractional saturation for the subunits are identical to the ones we wrote for the monomeric protein. However, we may not be satisfied with the above description, and we may ask how liganded and unliganded subunits are distributed within dimers, i.e. to what extent singly and fully liganded dimers are populated. In the absence of favorable or unfavorable interactions between monomers in the dimer, the distribution of liganded and unliganded subunits within dimers is expected to be statistical, i.e. binomial:

[fraction of unliganded subunits + fraction of liganded subunits]$^{\text{number of binding sites}}$ $= [\,(K\,/\,K+[L]) + ([L]\,/\,K+[L])\,]^2 = (\,K^2 + 2\,K\,[L] + [L]^2\,)\,/\,(K + [L])^2$

In the equation:

$$(K^2 + 2 K [L] + [L]^2) / (K + [L])^2$$

we recognize:
the fraction of unliganded dimers: $[P] / [P]_{tot} = K^2 / (K + [L])^2$;
that of singly liganded dimers: $[PL] / [P]_{tot} = 2 K [L] / (K + [L])^2$;
that of doubly liganded dimers: $[PL_2] / [P]_{tot} = [L]^2 / (K + [L])^2$

If we take the species [P] as a reference, and express all other species as a function of [P], we obtain:
$[PL] = [P]\, 2 [L] / K$
$[PL_2] = [P]\, [L]^2 / K^2$

which correspond to the binding polynomial and ligand saturation:

$$[P]_{tot} = [P]\, (1 + 2 [L] / K + [L]^2 / K^2) \qquad \text{(eqn. 4c)}$$

$$Y_L = \frac{2 [L] / K + 2 [L]^2 / K^2}{2 (1 + 2 [L] / K + [L]^2 / K^2)} = \frac{[L] / K}{1 + [L] / K} = \frac{[L]}{K + [L]} \qquad \text{(eqn. 4d)}$$

A dimeric protein which binds two molecules of the same ligand yields a population of ligation intermediates, but the overall fraction of bound sites remains a hyperbolic function of the ligand's concentration, unless some other condition occurs (e.g. intrinsic cooperativity).
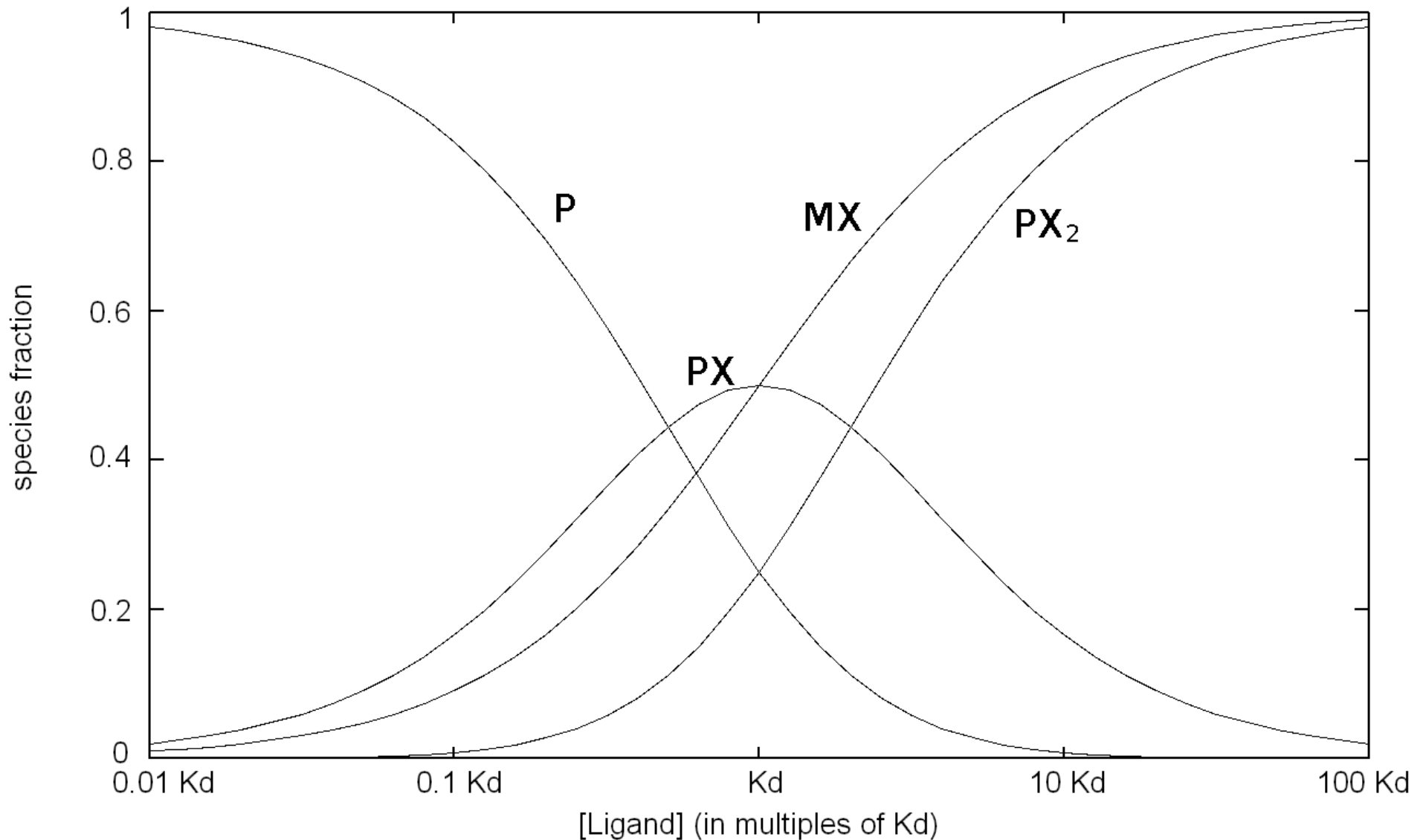
The *classical* thermodynamic view of cooperativity, formulated by Gilbert W. Adair in 1925, holds that "something" happens in the structure of the cooperative as a consequence of the binding of the first ligand, so that the affinity of the second ligand increases; i.e. the binding polynomial and ligand saturation functions of a symmetric homodimer become:

$$[P]_{tot} = [P] \left(1 + 2\,[L] / K_1 + [L]^2 / K_1 K_2\right) \qquad \text{(eqn. 4e)}$$

$$Y_L = \frac{2\,[L] / K_1 + 2\,[L]^2 / K_1 K_2}{2\left(1 + 2\,[L] / K_1 + [L]^2 / K_1 K_2\right)} \qquad \text{(eqn. 4f)}$$

And the ligand binding isotherm is sigmoidal, rather than hyperbolic if:
$$K_1 > K_2$$

Linus Pauling proposed in 1936 that in hemoglobin a liganded subunit forms a "facilitating" weak bond with the neighbouring ones, such that:
$$K_2 = K_1 / \alpha$$

Positive cooperativity implies that the partially liganded species are less populated than expected; negative cooperativity implies that the partially liganded species are more populated than expected.
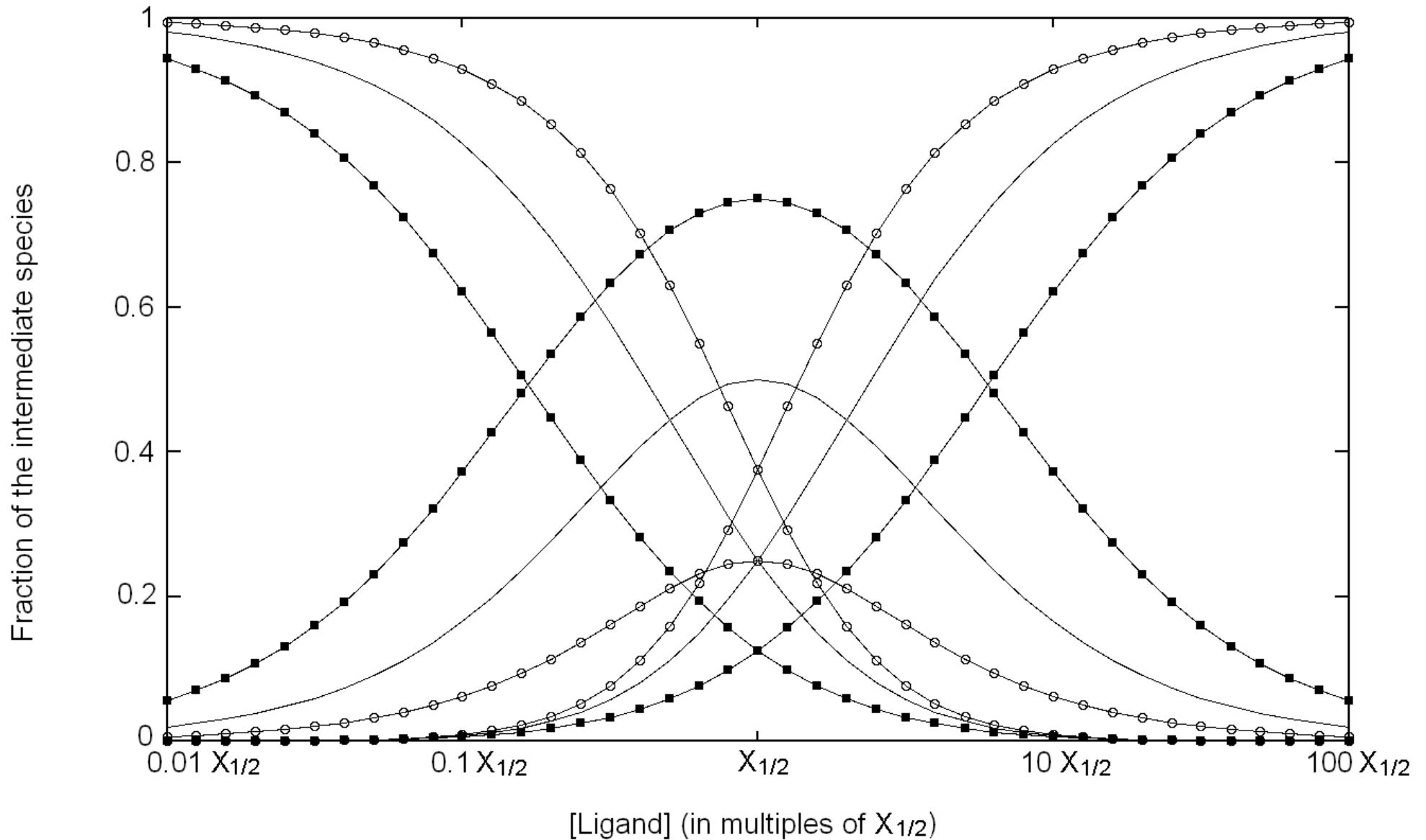
Vertebrate hemoglobins are tetramers (only a handful of Hbs from molluscs are cooperative homdimers)! As a consequence the binding polynomial according to Adair equation results:

$$[P]_{tot} = [P] \left(1 + 4\,[L]\,/\,K_1 + 6\,[L]^2\,/\,K_1K_2 + 4\,[L]^3\,/\,K_1K_2K_3 + [L]^4\,/\,K_1K_2K_3K_4\right) \quad \text{(eqn. 4g)}$$

$$Y_L = \frac{[L]\,/\,K_1 + 3\,[L]^2\,/\,K_1K_2 + 3\,[L]^3\,/\,K_1K_2K_3 + [L]^4\,/\,K_1K_2K_3K_4}{1 + 4\,[L]\,/\,K_1 + 6\,[L]^2\,/\,K_1K_2 + 4\,[L]^3\,/\,K_1K_2K_3 + [L]^4\,/\,K_1K_2K_3K_4}$$

and cooperativity arises if $K_1 > K_4$.

Cooperativity does not require that an ordered sequence $K_1 > K_2 > K_3 > K_4$ is present, but most "sequential" cooperativity models hypothesize orderly increasing cooperativity factors α, β, etc.

5. In a symmetric oligomer heterotropic linkage may be a cause of cooperativity.

What happens if we have heterotropic linkage in a homodimer? We shall consider only the simplest case, i.e. the thermodynamically symmetric homodimer, whose monomers are equivalent. Moreover the dimer is non cooperative for ligand L, both in the absence and in the presence of saturating amounts of ligand M:

$$
\begin{array}{ccccc}
 & K_L & & K_L & \\
P + L & \rightleftharpoons & PL + L & \rightleftharpoons & PL_2 \\
+ & & + & & + \\
M & & M & & M \\
K_M \updownarrow & & \updownarrow \; {}^{L}K_M & & \updownarrow \; {}^{LL}K_M \\
PM + L & \rightleftharpoons & PML + L & \rightleftharpoons & PML_2 \\
 & {}^{M}K_L & & {}^{M}K_L &
\end{array}
$$
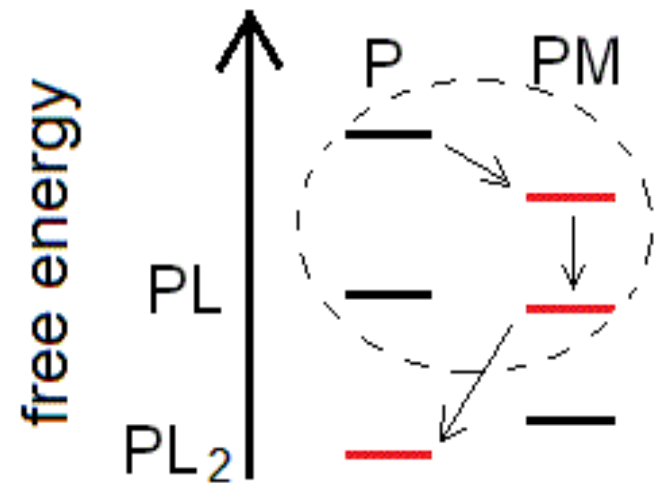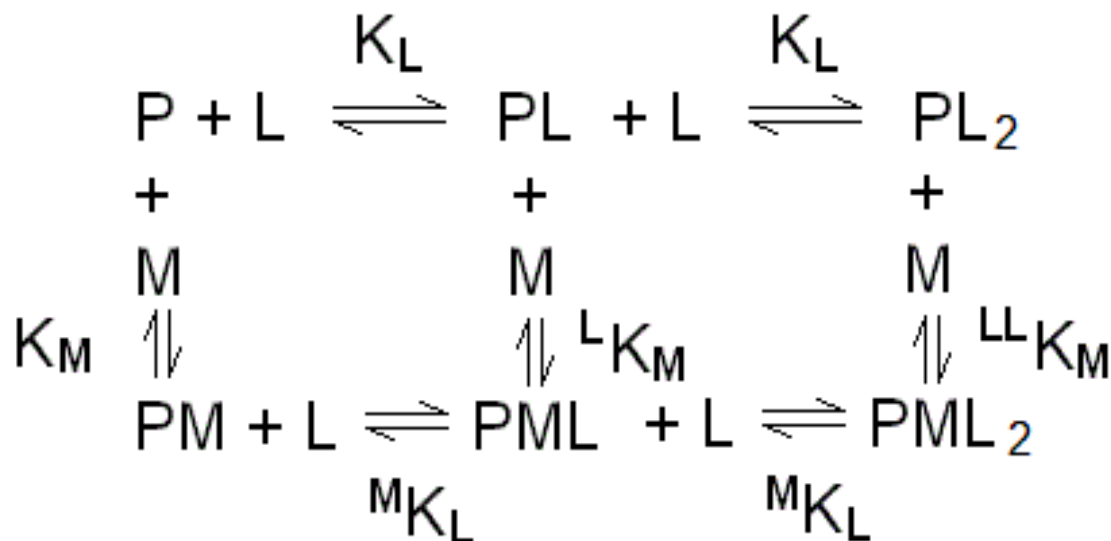
Because of the symmetry postulates we applied, the equilibrium constants are the same as in the case of the monomer, considered above (eqns. 3a to 3d), with the only addition of $^{LL}K_M$. The reason why we need this additional constant is that the system composed by the L- monoliganded and diliganded species forms a second thermodynamic square, analogous to the one described by (eqn. 3i):

$$^{L}K_M \ ^{M}K_L = K_L \ ^{LL}K_M \qquad \text{or} \qquad ^{LL}K_M = {}^{L}K_M \ ^{M}K_L / K_L = K_M \left({}^{M}K_L / K_L \right)^2$$

We also remark that the energy profile is again depicted for the case of negative heterotropic linkage, and that depending of the concentrations of [M] and [L], the preferred species would be those highlighted in red, with the order of appearance indicated by the arrows. Thus, if we start with the protein and nothing else in solution we only have P; when we add M, PM becomes populated; and as we increase the concentration of L we populate first PML and next PL_2, while M is released. The dashed circle indicates the area of the energy profile which corresponds to the one considered in the above section 3.

The binding polynomial of our system is:

$$[P]_{tot} = [P] [(1 + [L]/K_L)^2 + [M]/K_M (1 + [L]/^M K_L)^2]$$ (eqn. 5a; cfr. 3j)

A graphical representation of our system, which highlights the symmetry of the two binding sites is as follows:
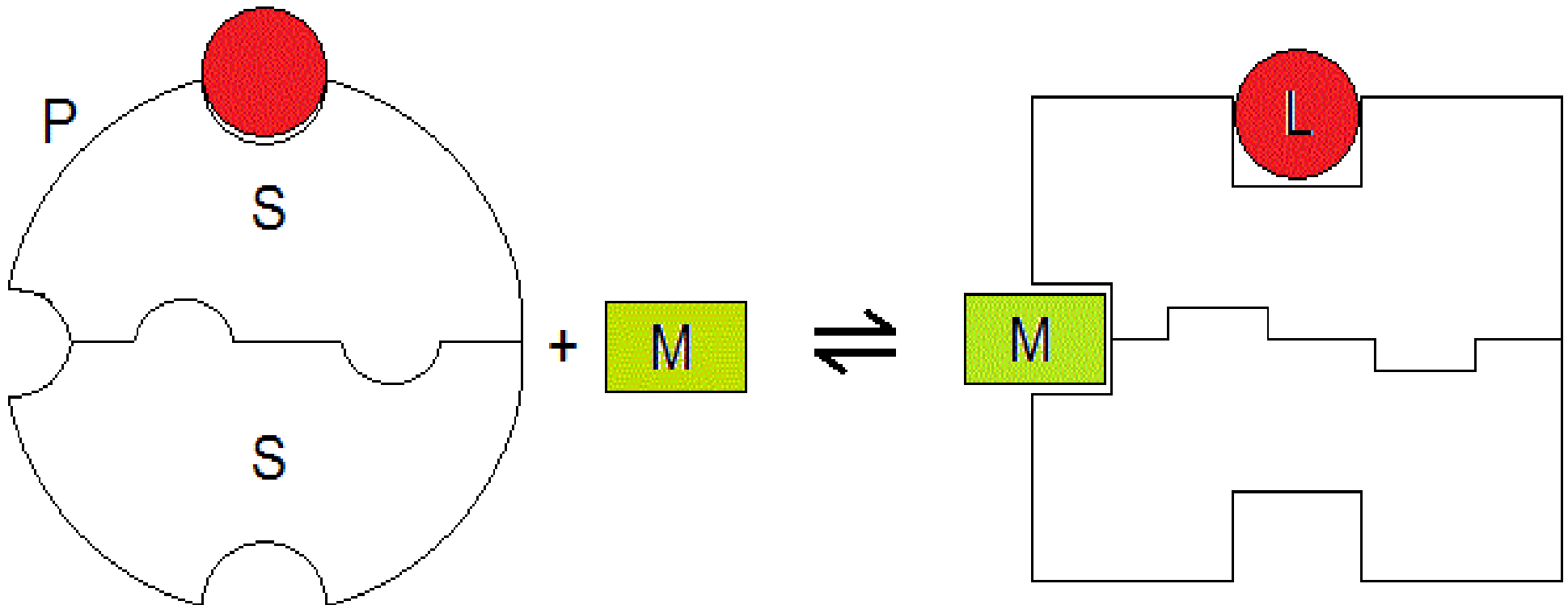
The fraction of ligand saturation for ligand L results:

$$Y_L = \frac{2\,[L]/K_L\,(1 + [L]/K_L) + 2\,[M][L]\,/\,K_M\,^{M}K_L(1 + [L]/^{M}K_L)}{2\,(1 + [L]/K_L)^2 \;+\; 2\,[M]/K_M\,(1 + [L]/^{M}K_L)^2} \quad \text{(eqn. 5b)}$$

Under appropriate experimental conditions, eqn. 5b (to be compared with 3k), yields a sigmoid, i.e. *cooperative*, binding isotherm.

One may ask how the linked heterotropic ligand M can introduce positive homotropic cooperativity for ligand L in the protein; but this question is ill conceived: *cooperativity is the consequence of symmetry*, not of the binding of M. The ligand M only makes the relationship between symmetry and cooperativity evident.

Take home message #6: *heterotropic linkage may induce cooperativity in an otherwise non-cooperative oligomeric protein, if the symmetry requirement is fulfilled.*
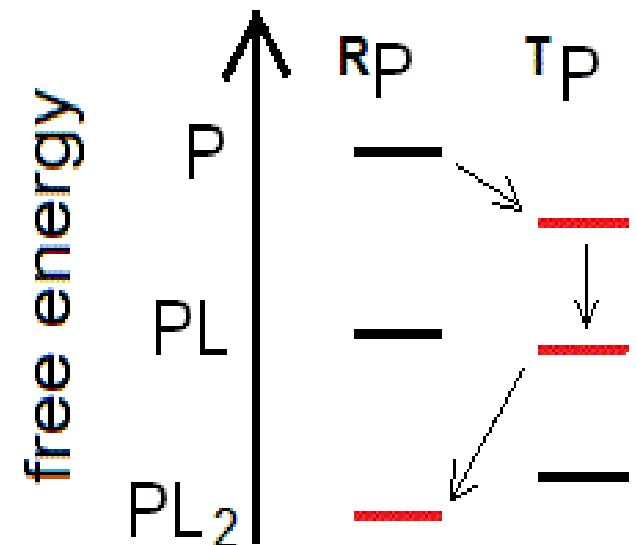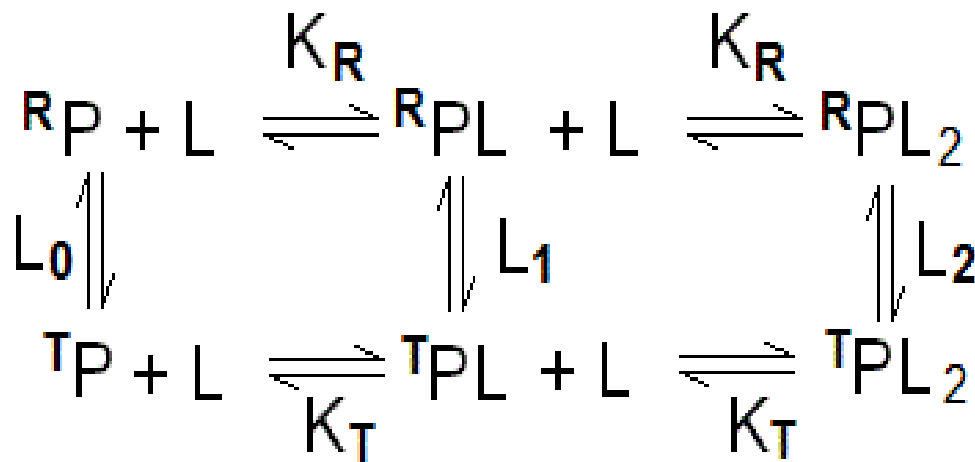
6. Does hemoglobin need the heterotropic effector? The two-state allosteric model by Monod, Wyman and Changeux.

In 1963 J. Monod coined the concept of allostery (greek: other structure) to describe a protein stable in two different three dimensional structures. Monod's interest at the time was focussed on the heterotropic regulation of enzymes; but shortly afterward he was to discover another case of allosteric regulation, namely the repressor protein of the operon Lac (for which he was awarded the Nobel Prize for Medicine and Physiology).

If Wyman's interpretation of heterotropic linkage was based on a pure, disembodied thermodynamic formalism, Monod's one had a sort of esthetical connection to the shape of proteins, as real objects, and his key point was about molecular symmetry. *Monod was fascinated by what he called "nature's elegance" and he is reported to have said that elegance does not prove a model true, but lack of elegance certainly proves it false.*

The allosteric model postulates that the protein is a symmetric oligomer, made up of identical (or at least equivalent) subunits, stable in two different quaternary conformations (called R and T) with different affinities for the ligand.

Allostery in a protein oligomer yields a thermodynamic system similar to the one considered in the preceding section, with one important difference: the two structural states are to be considered as conformational isomers of the protein, that freely equilibrate in the absence of any ligand. The reaction pathway and energy profile of ligand binding to an allosteric homodimer is as follows:

$$^RP + L \xrightleftharpoons{K_R} {}^RPL + L \xrightleftharpoons{K_R} {}^RPL_2$$

$$\Big\updownarrow L_0 \qquad\qquad \Big\updownarrow L_1 \qquad\qquad \Big\updownarrow L_2$$

$$^TP + L \xrightleftharpoons[K_T]{} {}^TPL + L \xrightleftharpoons[K_T]{} {}^TPL_2$$

The binding polynomial for this system is the sum of the two binding polynomials of the R and T state, weigthed by the allosteric constant L_0, which describes the isomerization equilibrium R<==>T in the absence of the ligand. For a homodimer:

$$[P]_{tot} = [R_0] [(1 + [L] / K_R)^2 + L_0 (1 + [L] / K_T)^2] \qquad (eqn. 6a)$$

To generalize to a n-subunit protein we only need to substitute the exponent with the number of binding sites: e.g. for a tetrameric hemoglobin:

$$[P_{tot}] = [R_0] [(1 + [L]/K_R)^4 + L_0 (1 + [L]/K_T)^4] \qquad (eqn. 6b)$$

The fraction of liganded sites for a n-subunit protein results:

$$Y = \frac{[L]/K_R (1 + [L]/K_R)^{n-1} + L_0 [L]/K_T (1 + [L]/K_T)^{n-1}}{(1 + [L]/K_R)^n + L_0 (1 + [L]/K_T)^n} \qquad (eqn. 6c)$$

Key features of the two-state model include:

(i) cooperative proteins are symmetric oligomers stable in two (tertiary and quaternary) conformations called T and R.

(ii) The T- and R- states/structures are both populated and in equilibrium with each other both in the presence and in the absence of the ligand; they should be considered as conformational isomers.

(iii) The T-state is predominant in the absence of the ligand and has low affinity; the R-state is predominant in the presence of excess ligand and has high affinity. At some point in the ligand binding isotherm the predominant state must change (*switch-over point*).

(iv) The T- and R- states are fully symmetric and non-cooperative. Cooperativity is a consequence of the ligand-dependent structure change.

(v) All ligation intermediates are fully symmetric and either have the T- or the R-structure. No intermediate structures exist and no state exists in which T-like and R-like subunits are mixed.

7. The structure of hemoglobin: a primer.

The widespread adoption of the two-state model to explain hemoglobin cooperativity goes beyond its simplicity and its ability to explain a huge array of experimental data, and refers to its almost incredible predictive capabilities and its striking adherence to structural details discovered long after its formulation.

Hemoglobin is a tetramer made up of two types of subunits called α and β. In order to name the four subunits the notation $\alpha_1, \alpha_2, \beta_1$ and β_2 is universally adopted. The subunits' structure is very similar (the "globin fold") and consists of 8 α-helices named A thorugh H plus short inter-helical segments. The interfaces between the subunits are of the type that Monod called **isologous**, i.e. symmetrical. Each α-subunits contacts the two βs and vice-versa, with the pseudo-symmetric contact regions:

α_1H-β_1B, α_1G-β_1G, and α_1B-β_1H for the $\alpha_1\beta_1$ interface
and
α_1C-β_2FG, and α_1FG-β_2C for the $\alpha_1\beta_2$ interface.

The $\alpha_1\beta_1$ interface of human hemoglobin.
Red/purple: helices A,B; green C,D,E,F; yellow/orange: G,H
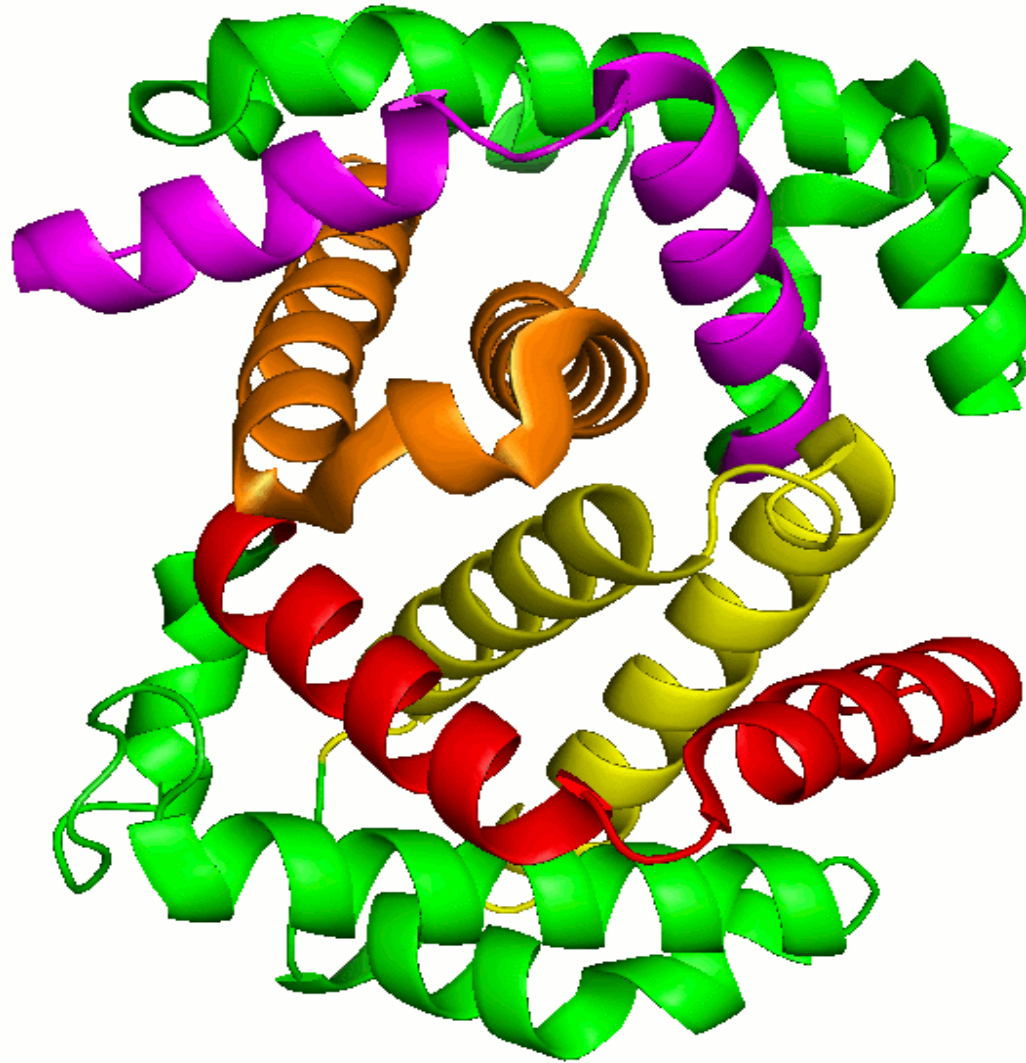
A schematic representation of the two-states allosteric model, as applied to hemoglobin is as follows (notice that the allosteric interface, where the ligand-dependent structural change occurs is symmetric and isologous):
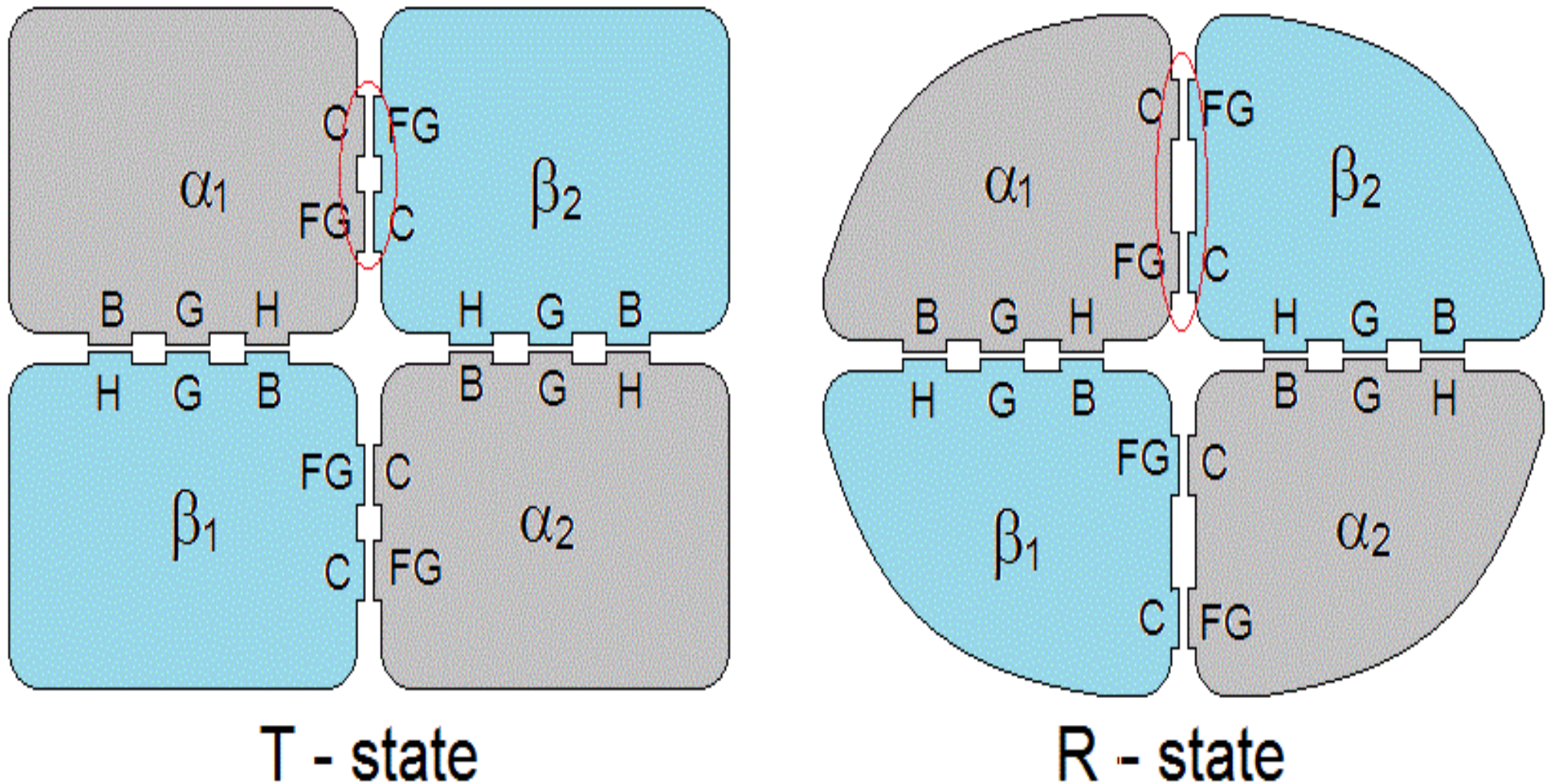
T - state

R - state

A short list of some experimental data on hemoglobin that strongly support the two-state allosteric model or some of its variants.

(i) Only two families of 3D structures have been discovered by X-ray crystallography: the low affinity (T) family with very limited variability, and the high affinity R family, with greater variability (structures R, R_2, R_3, etc.).

(ii) The structures of the "poorly populated" state $^{T}Hb(O_2)_4$ has been solved, whereas the other poorly populated state (unliganded ^{R}Hb) up to now has only been obtained by chemical modification or site directed mutagenesis.

(iii) The structures of the diliganded ligation intermediates $\alpha_2^{CO}\beta_2$ and $\alpha_2\beta_2^{CO}$ have been obtained; these are unequivocally T-like, and no intermediate has been crystallized in an intermediate, neither T-nor R structure; moreover no intermediate presents an asymmetric structure at the interfaces.

(iv) The kinetic rate constants for the allosteric quaternary structural change have been measured, taking advantage of structure-specific spectroscopic features; these satisfactorily agree with those calculated from equilibrium binding experiments.

(v) Cooperativity is abolished if ligand binding experiments are carried out under conditions in which the quaternary structural change is prevented (e.g. in crystals; in silica gels). Hemoglobin blocked in the R-conformation has high affinity, in the T-conformation it has low affinity. The same applies to chemically modified, site directed mutant, and CP digested hemoglobins.

Experimental results on hemoglobin that require an extension of the two-state model.

In spite of its success in explaining and predicting the functional behaviour of hemoglobin, the two-state model also has well known defects and failures, that have required its amendement:

(i) in its original formulation the model ascribed heterotropic effects to the preferential binding of the effector to either the T or the R state; as a consequence it predicted that heterotropic effects would be limited to variations of the allosteric constant L_0. However, it has been demonstrated long ago that both K_R and K_T depend on heterotropic effectors, and thus require an extension of the model.

(ii) The time course of oxygen dissociation from $^T HbO_2$ cannot be fitted to one exponential, an observation that was riginally explained invoking the functional inequivalence of the α and β subunits. Successive experiments have demonstrated that the functional inequivalence of the subunits is minimal, thus this result is at present unexplained.

Conclusions

Hemoglobin is prototype model of cooperativity, in which we recognize:

(i) an **intrinsic** or **true** cooperativity present also under experimental conditions in which allosteric effectors are either absent or present at concentrations insufficient to cause significant binding (e.g. pH = 9 in the absence of chloride, CO_2 and phosphates). K. Imai reports for these conditions a $P_{1/2\ O2}$ of 1 mmHg, a Hill coefficient of 2.35 and a ratio K_1 / K_4 (or K_T / K_R) = 22. Under these experimental conditions, Hb seems to obey a pure two-state model.

(ii) Under physiological conditions cooperativity is enhanced by heterotropic effects, which add a quota of **extrinsic** or **pseudo-**cooperativity, due to preferential binding to the T-state and to a direct effect on K_T. E.g. Imai reports $P_{1/2\ O2}$ of 14 mmHg, a Hill coefficient of 3.1 and a ratio K_1 / K_4 (or K_T / K_R) = 470 at physiological pH, 0.1 M chloride and 2 mM DPG.