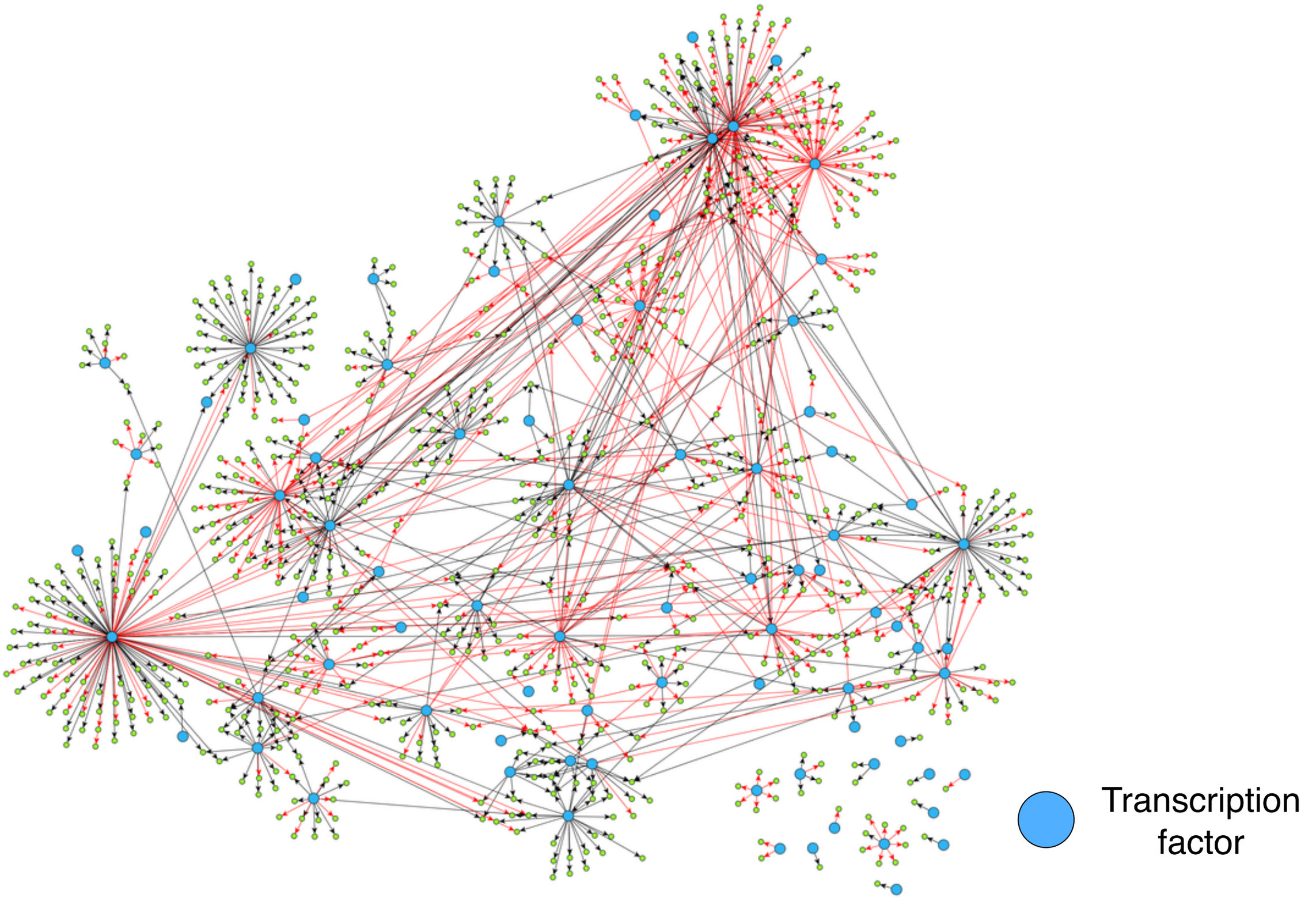
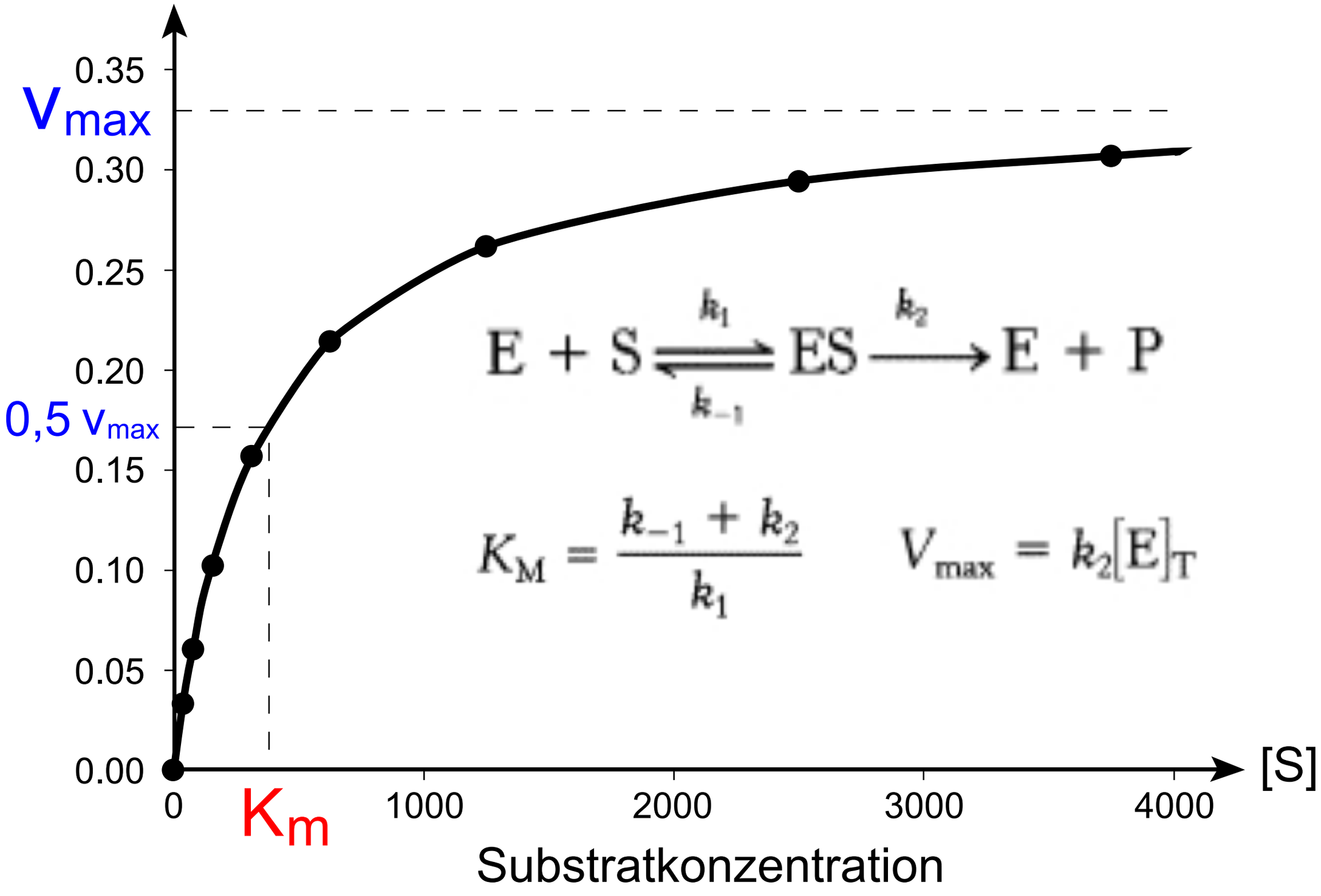
What is the basis of regulatory transcription factor networks?

Interaction affinity, kinetic rates and associated enzymatic reactions



Michaelis-Menten enzyme kinetics: reaction rate V in dependence of substrate concentration



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

$$K_M = \frac{k_{-1} + k_2}{k_1} \qquad V_{max} = k_2[E]_T$$

Increasing complexity of equilibrium binding descriptions

all binding sites are
equivalent and independent

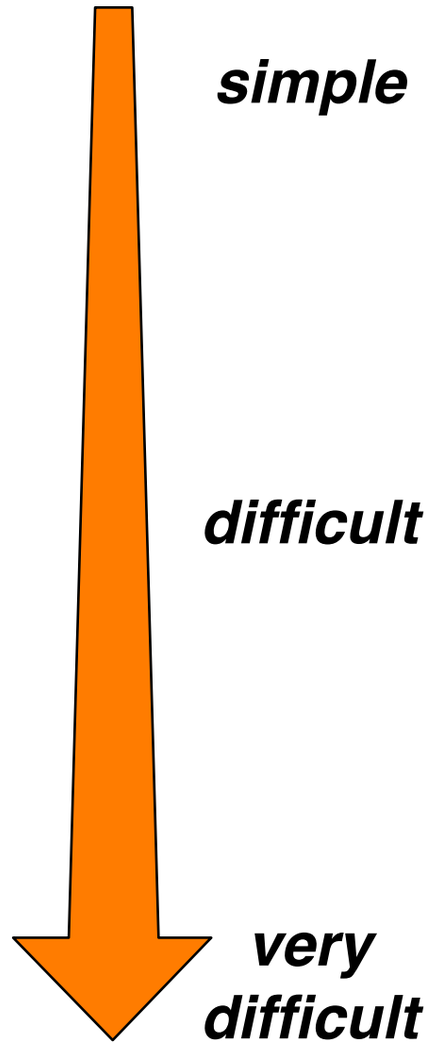
cooperativity ↙ ↘ ***heterogeneity***

all binding sites are
equivalent and not independent

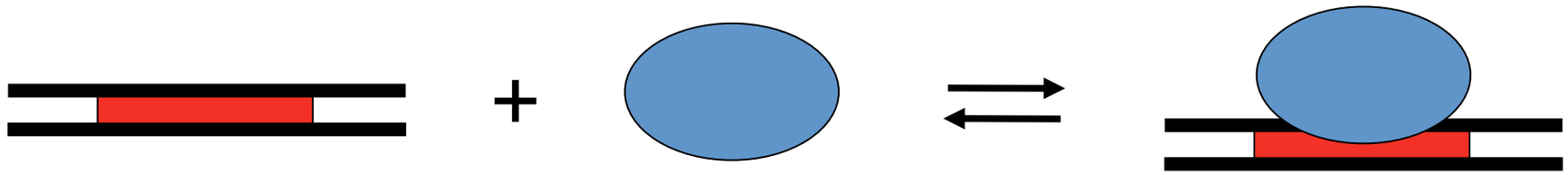
all binding sites are
independent but not equivalent

heterogeneity ↘ ↙ ***cooperativity***

all binding sites are
not equivalent and not independent



The mass equation law for binding of a protein “P” to a DNA “D” with a single binding site



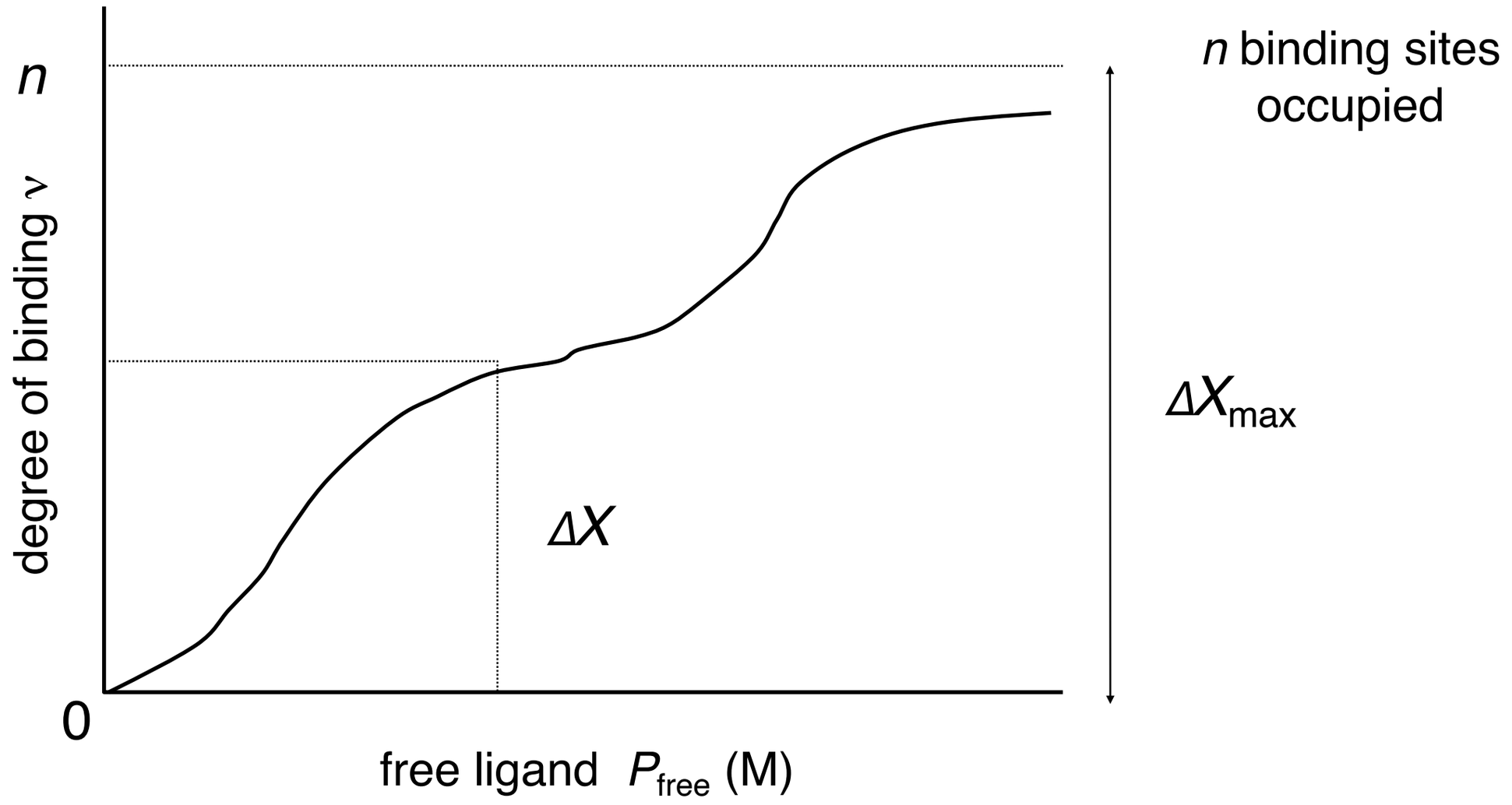
$$D_{\text{free}} + P_{\text{free}} \underset{k_{\text{off}}}{\overset{k_{\text{on}}}{\rightleftharpoons}} DP \qquad K_1 = \frac{D_{\text{free}} \cdot P_{\text{free}}}{DP} = \frac{k_{\text{off}}}{k_{\text{on}}}$$

binding of the first proteins with the dissociation constant K_1

D_{free}, concentration free DNA; P_{free}, concentration free protein

$$\text{binding constant } K_{\text{B}} = \frac{1}{\text{dissociation constant } K_{\text{D}}}$$

Titration of a macromolecule *D* with *n* binding sites for the ligand *P* which is added to the solution



$$\frac{\Delta X}{\Delta X_{\max}} = \frac{\nu}{n} = \theta \text{ (fraction saturation)} \qquad \nu = \frac{[\text{bound ligand } P]}{[\text{macromolecule } D]}$$

Schematic view of gel electrophoresis to analyze protein-DNA complexes

Mark M. Garner and Arnold Revzin

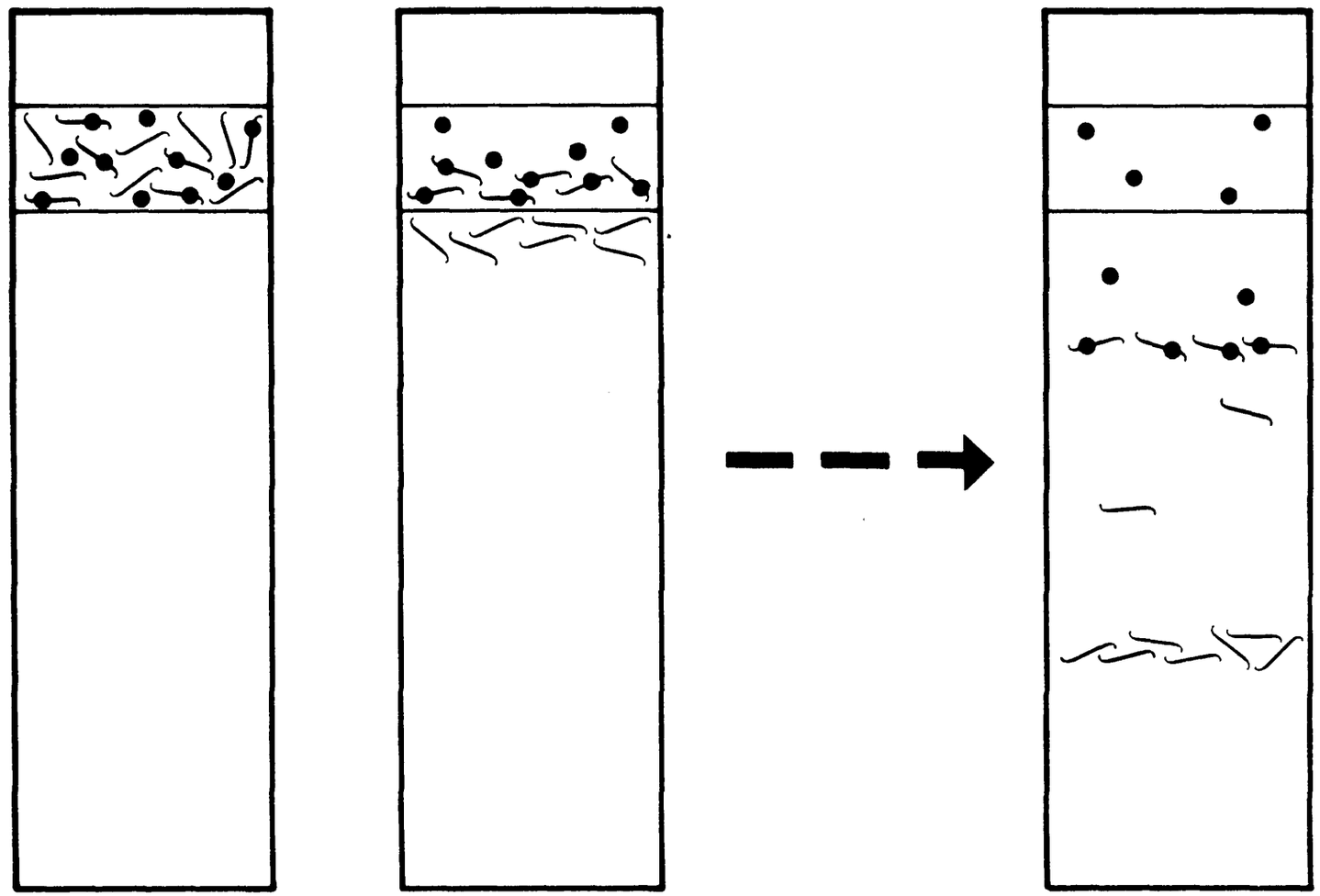
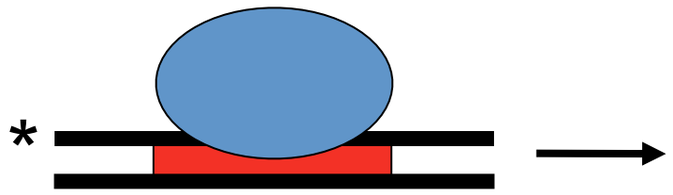
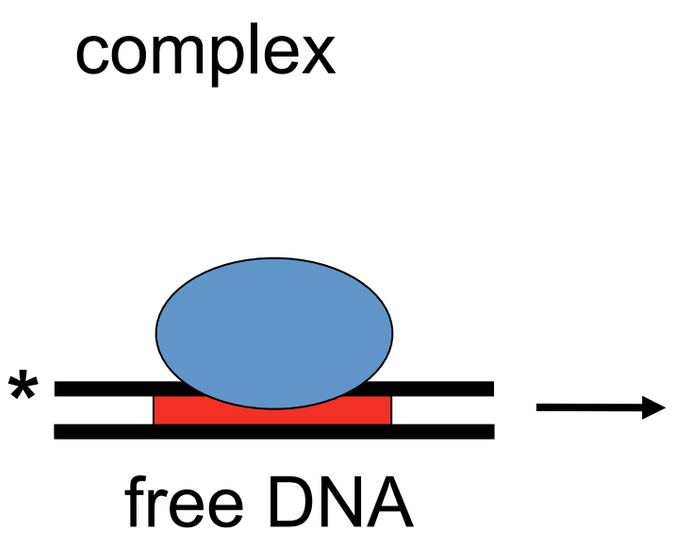


Figure 1. Schematic diagram of the gel retardation method. The filled circles represent protein, curved lines represent linear DNA fragments. The left-hand panel illustrates the DNA – protein solution loaded on to the gel. The middle panel shows free DNA entering the gel just after the power is turned on. The right-hand panel depicts the situation later in the run; bands of complexes and of free DNA are seen. If the complexes dissociate during electrophoresis, the DNA released never catches up with the main band of free DNA.

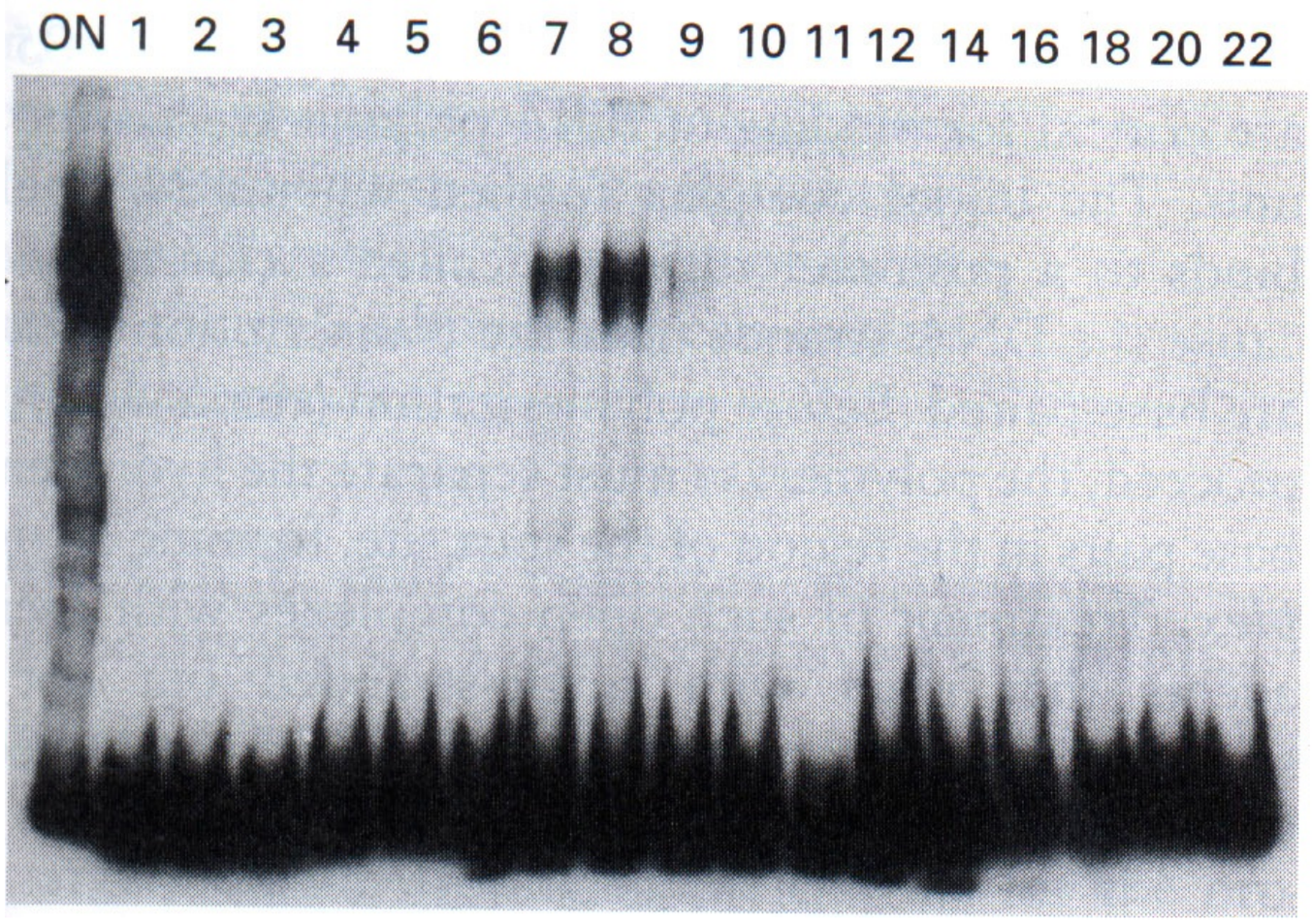
“Gel shift”: electrophoretic mobility shift assay (“EMSA”) for DNA-binding proteins



Protein-DNA complex



free DNA



1. Prepare labeled DNA probe
2. Bind protein
3. Native gel electrophoresis

Advantage: sensitive, fmol DNA

Disadvantage: requires stable complex; little “structural” information about which protein is binding

EMSA of Lac repressor binding to operator DNA
From (a) to (j) the concentration
of lac repressor is increased.

Complexes with

Free DNA

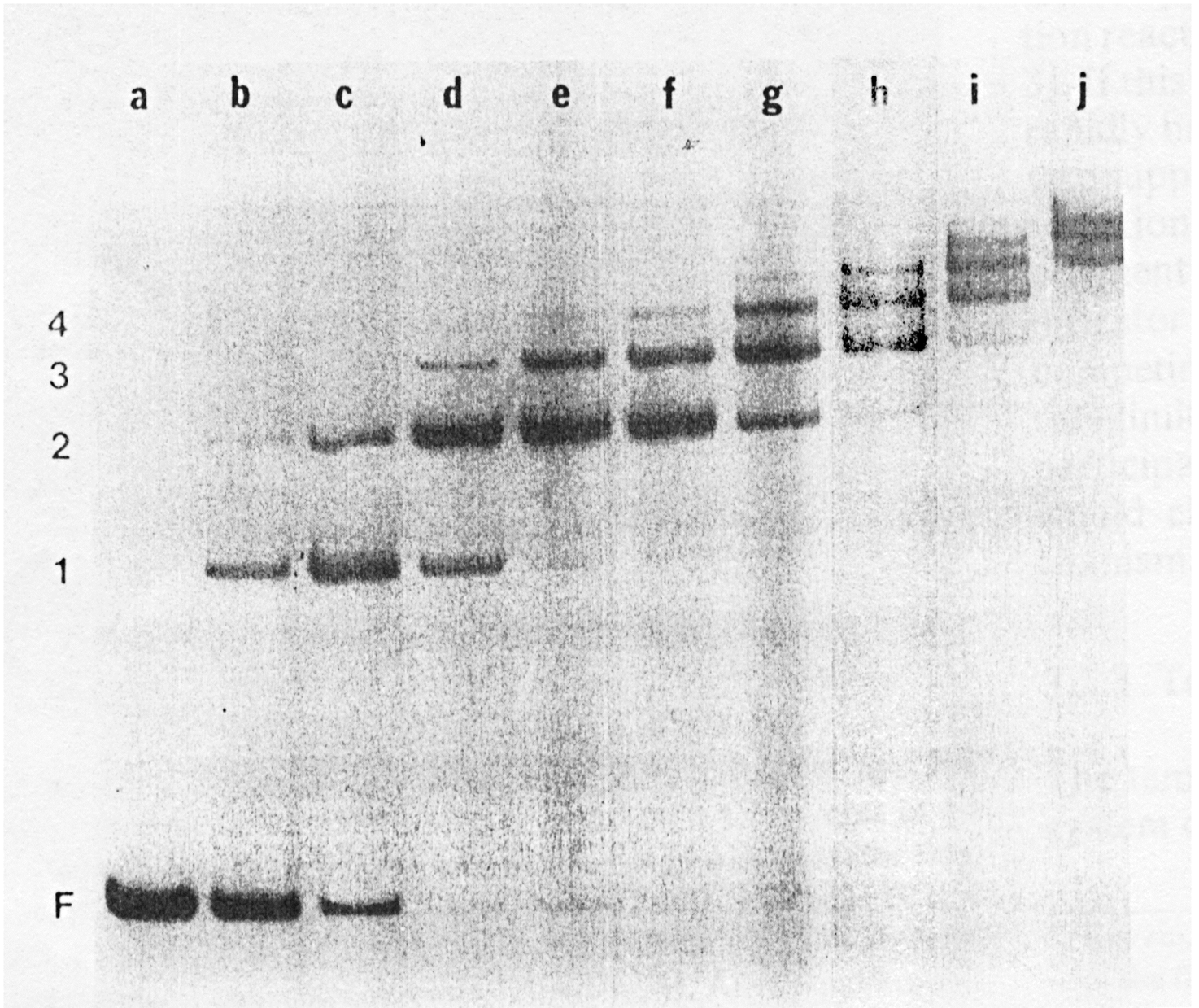
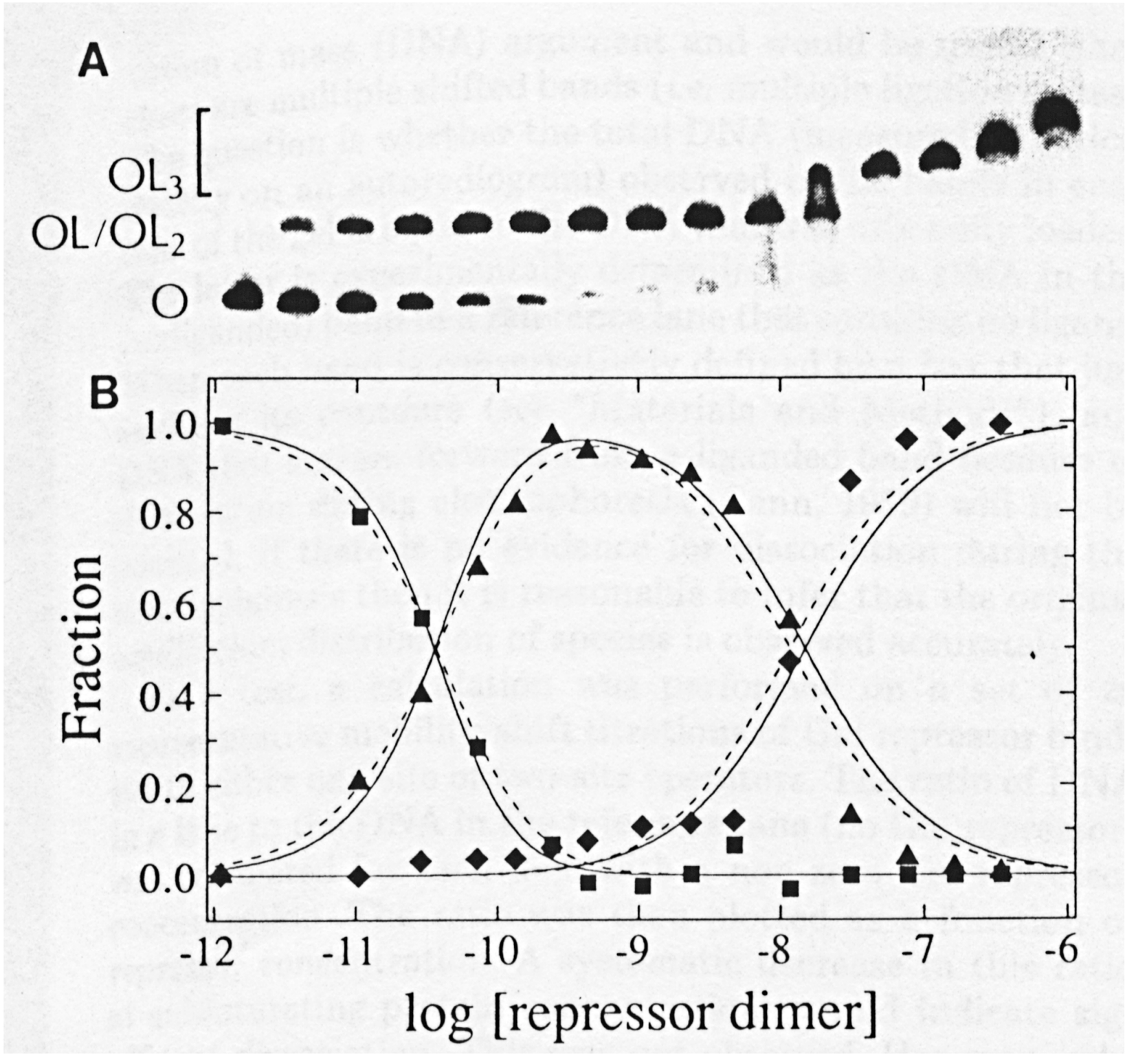
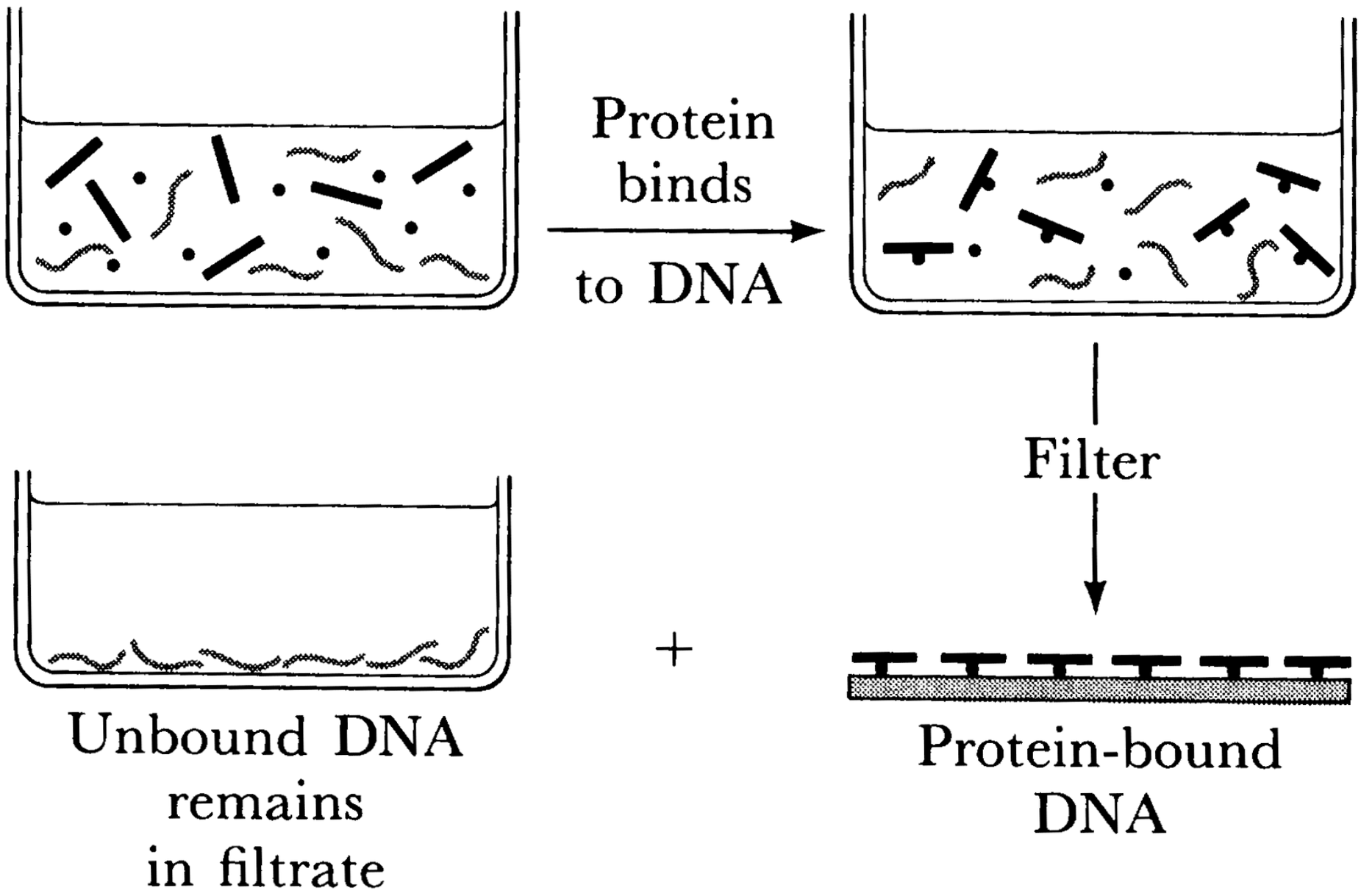


Figure 1. Titration of the 203 bp L8-UV5 lactose promoter-operator fragment with *lac* repressor. The DNA fragment concentration was 18.5 nM *Lac* repressor concentrations were: 0, 12.4, 24.8, 37.0, 49.4, 61.6, 74.0, 98.8, 123.4 and 148.0 nM for samples (a)–(j) respectively. Samples were incubated for 30 min at room temperature in 10 mM Tris (pH 8.0 at 21 °C), 1 mM EDTA, 50 mM KCl, and applied to a 5 % polyacrylamide gel equilibrated with the same buffer. Electrophoresis was at 8 V/cm for 2 h. The repressor: fragment ratios [1] of some complexes are given of the left margin. Band F denotes free DNA.

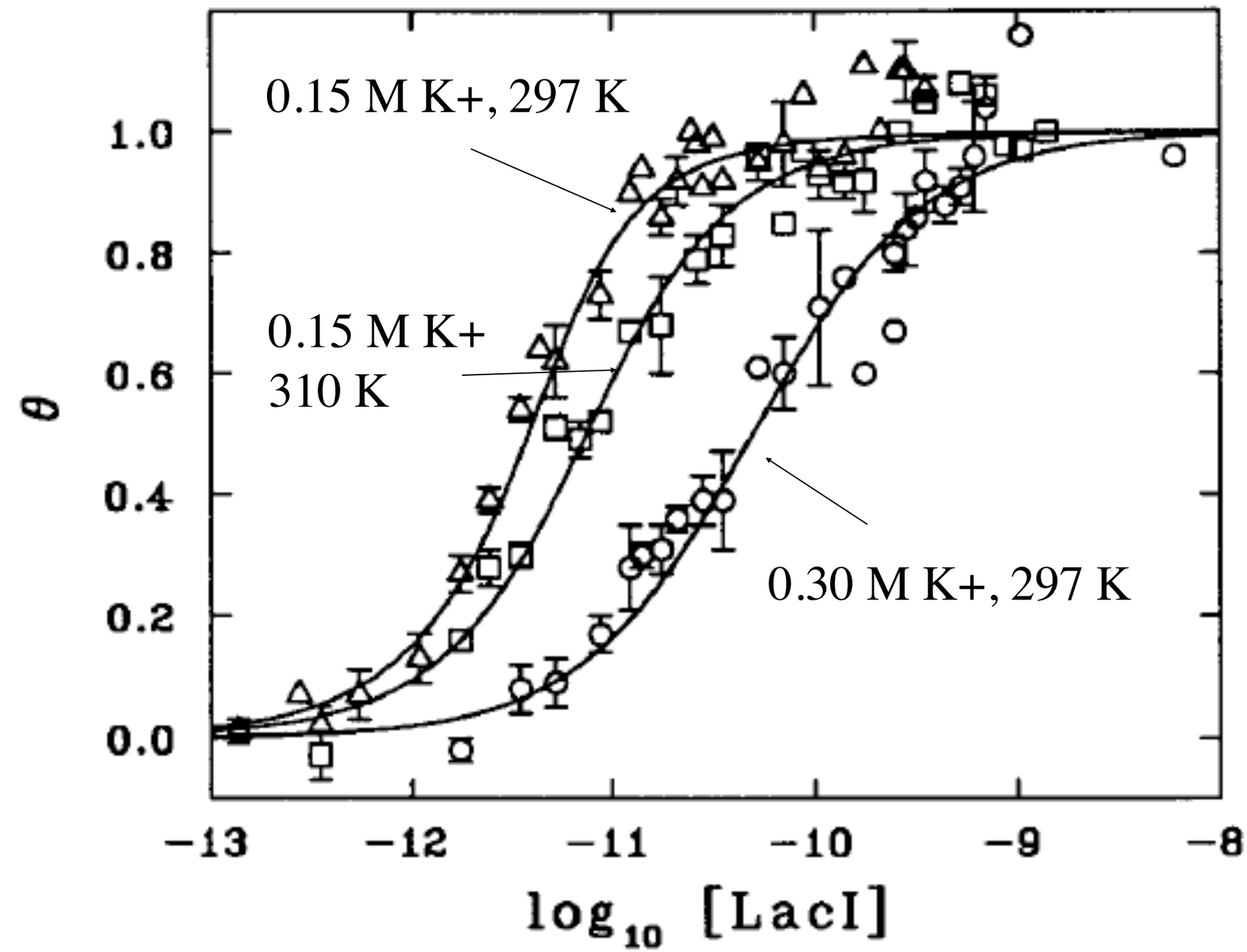
Measuring binding constants for lambda repressor on a gel



Principle of filter-binding assay

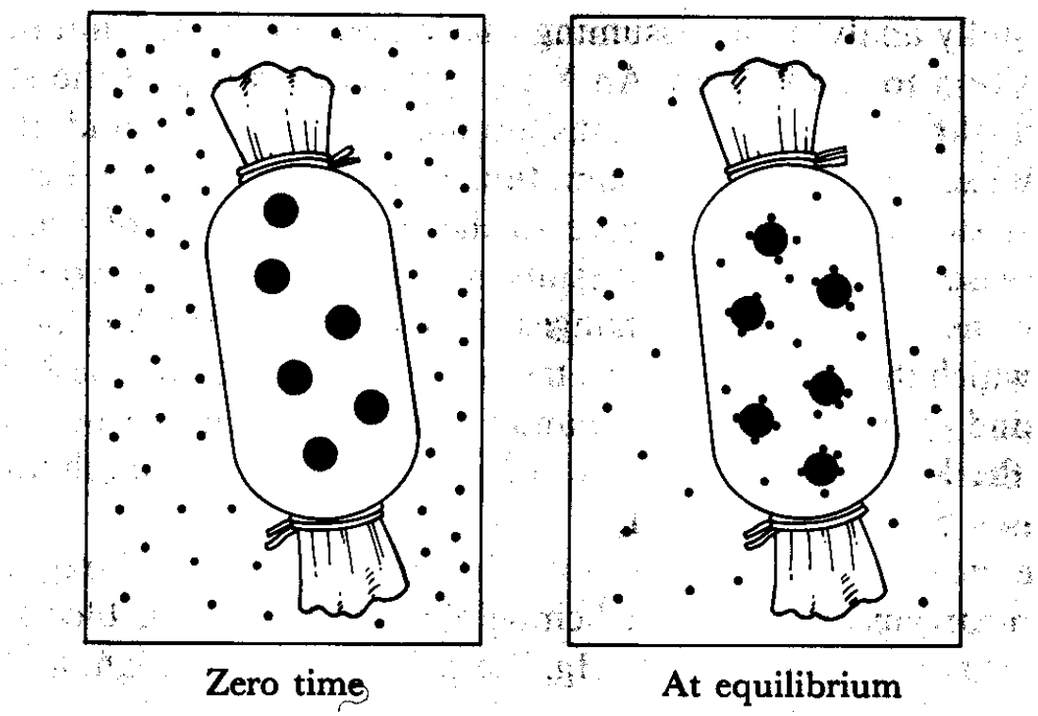


Binding titrations of symmetrical operator site with Lac repressor measured by filter binding assay



Binding measurements by equilibrium dialysis

A macromolecule is dialyzed against a solution of ligand. Upon reaching equilibrium, the ligand concentration is measured inside and outside the dialysis chamber. The excess ligand inside the chamber corresponds to bound ligand.



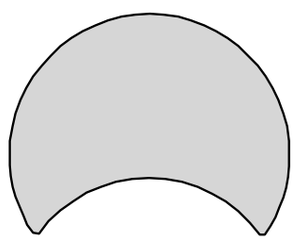
$$\nu = \frac{[X]_{in} - [X]_{out}}{M}$$

- direct measurement of binding

-non-specific binding will obscure results, work at moderate ionic strength (≥50 to avoid the *Donnan Effect* (electrostatic interactions between the macromolecule and a charged ligand.

- needs relatively large amounts of material

Analysis of binding of RNAP·σ^{54} to a promoter DNA sequence by measurements of fluorescence anisotropy



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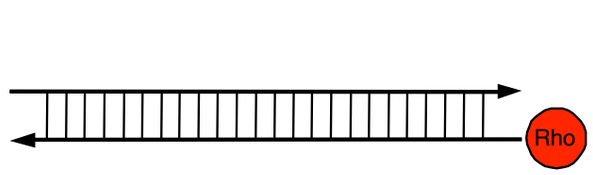
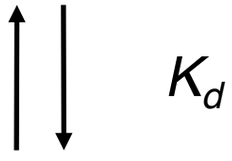
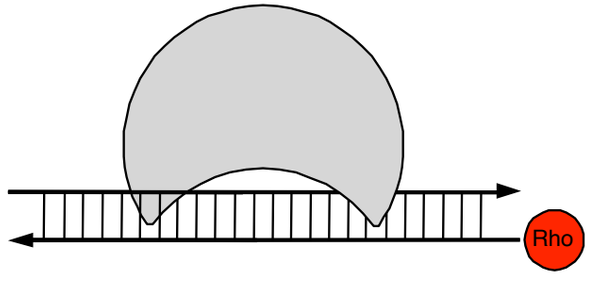


promoter DNA

free DNA with a fluorophore
with high rotational diffusion
-> low fluorescence anisotropy r_{min}

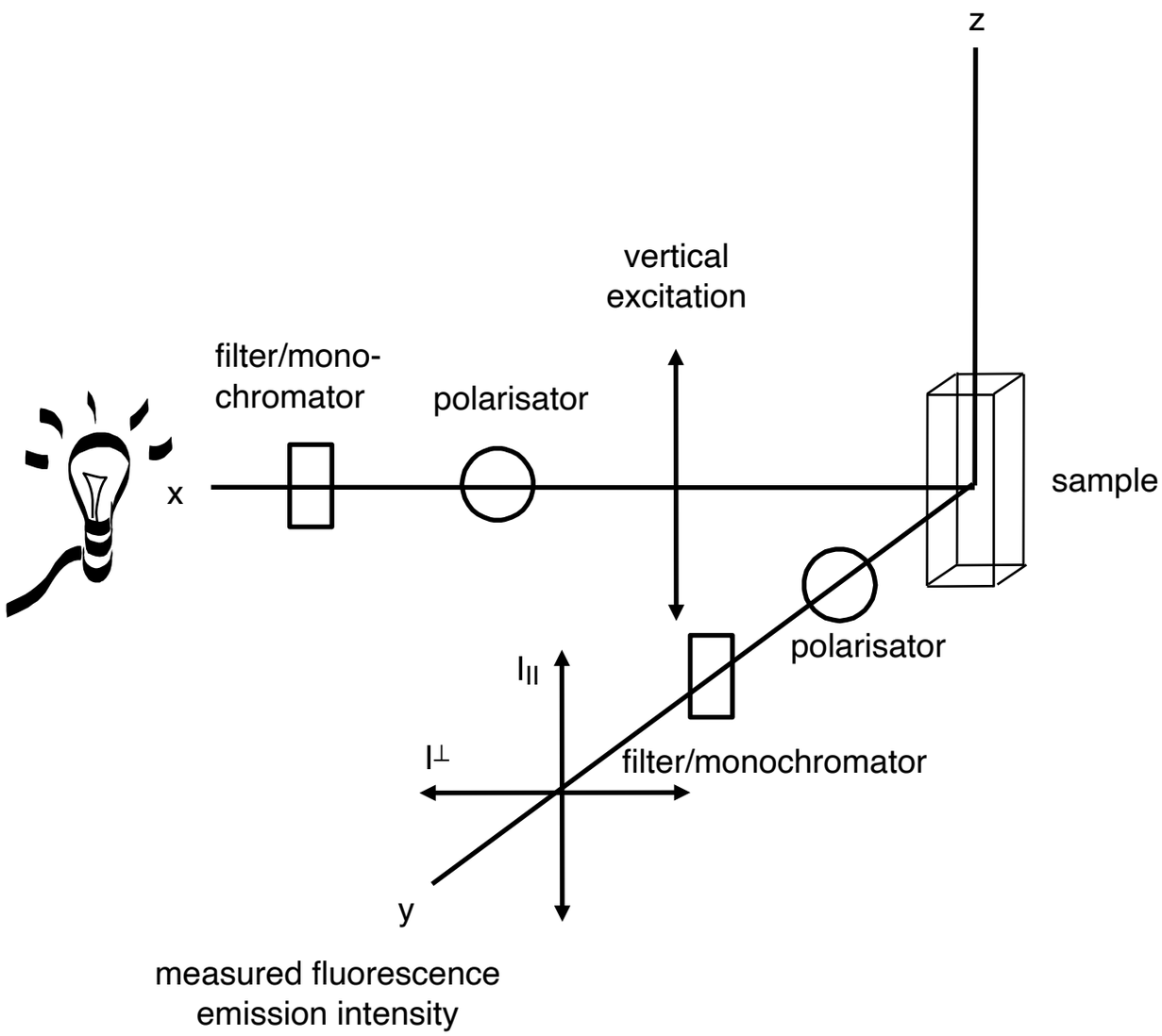




RNAP·σ^{54}-DNA-Komplex

RNAP-DNA complex
with low rotational diffusion
-> high fluorescence anisotropy r_{max}

How to measure binding of a protein to DNA?
One possibility is to use fluorescence anisotropy

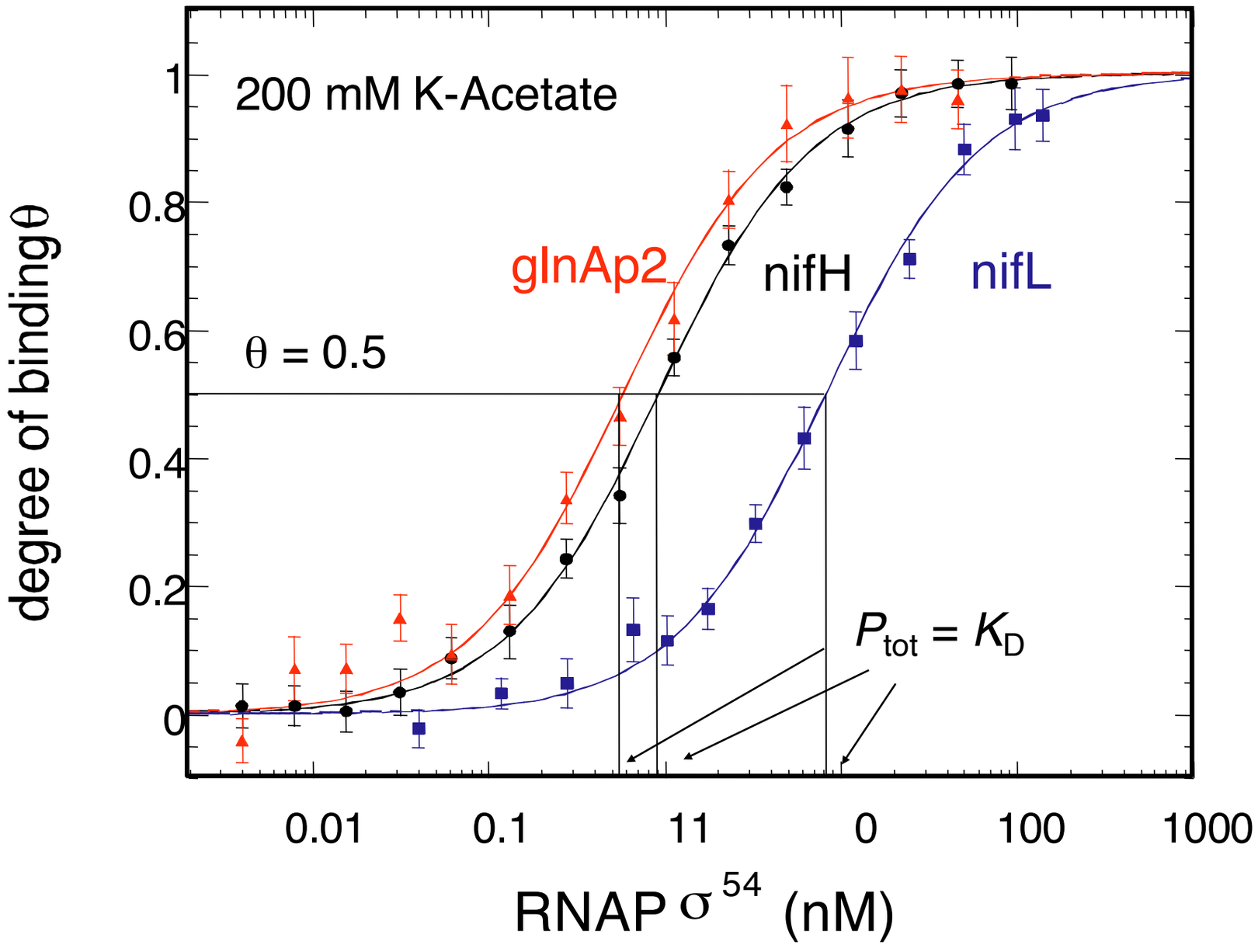


$$r = \frac{I_{II} - I_{\perp}}{I_{II} + 2I_{\perp}}$$

Definition of fluorescence anisotropy *r*

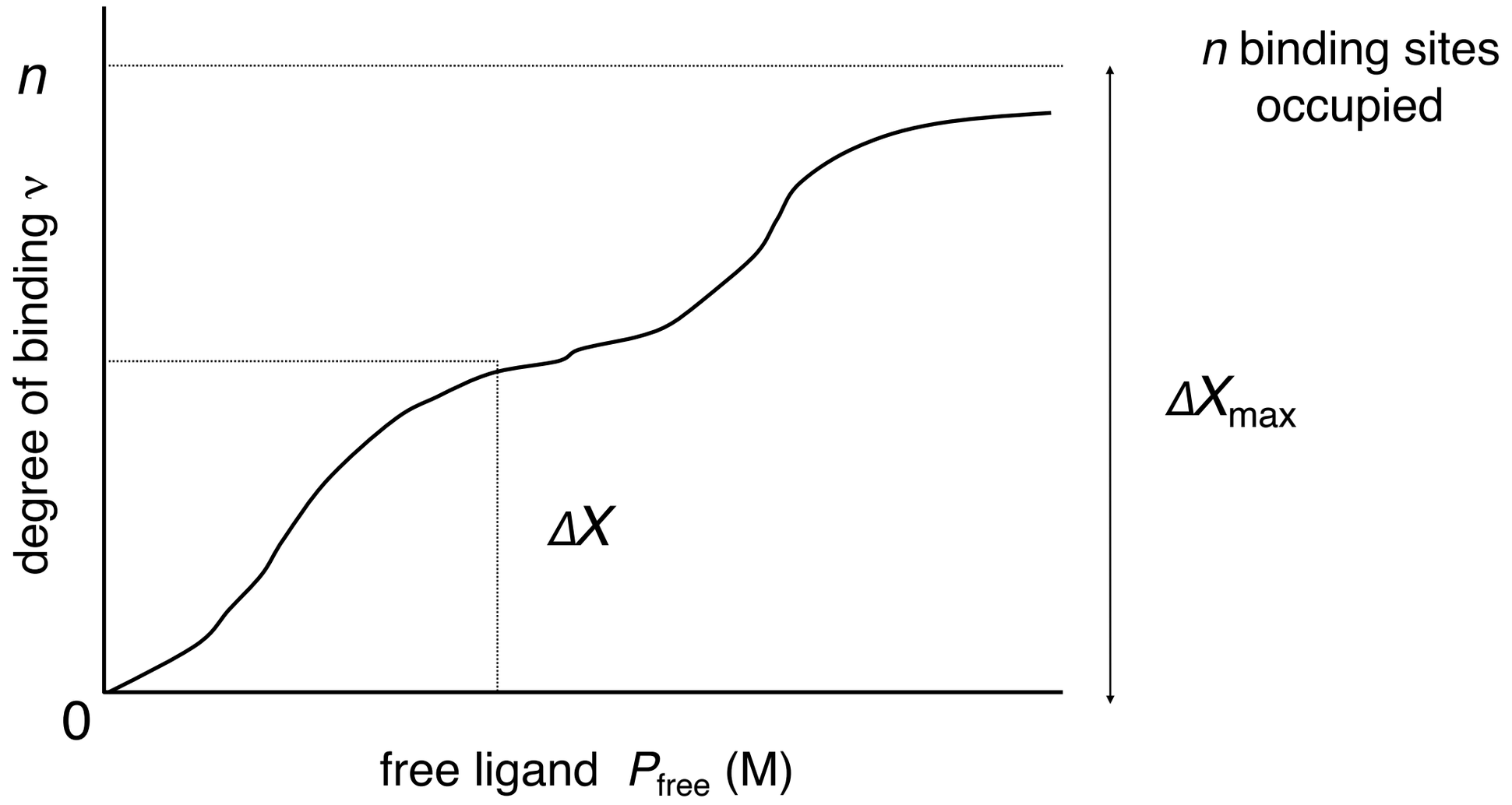
The anisotropy *r* reflects the rotational diffusion of a fluorescent species

Measurements of fluorescence anisotropy to monitor binding of RNAP·σ^{54} to different promoters



Vogel, S., Schulz A. & Rippe, K.

Titration of a macromolecule *D* with *n* binding sites for the ligand *P* which is added to the solution



$$\frac{\Delta X}{\Delta X_{\max}} = \frac{\nu}{n} = \theta \text{ (fraction saturation)} \qquad \nu = \frac{[\text{bound ligand } P]}{[\text{macromolecule } D]}$$

Example: binding of a protein *P* to a DNA-fragment *D* with one or two binding sites

$$D_{\text{free}} + P_{\text{free}} \underset{k_{\text{off}}}{\overset{k_{\text{on}}}{\rightleftharpoons}} DP \qquad K_1 = \frac{D_{\text{free}} \cdot P_{\text{free}}}{DP} = \frac{k_{\text{off}}}{k_{\text{on}}}$$

binding of first proteins with dissociation constant K_1

D_{free}, concentration free DNA; P_{free}, concentration free protein;

DP, complex with one protein; DP_2, complex with two proteins;

$$DP + P_{\text{free}} \rightleftharpoons DP_2 \qquad K_2 = \frac{DP \cdot P_{\text{free}}}{DP_2}$$

binding of second proteins with dissociation constant K_2

$$D + 2P_{\text{free}} \rightleftharpoons DP_2 \qquad K_2^* = \frac{D_{\text{free}} \cdot P_{\text{free}}^2}{DP_2} \qquad K_2^* = K_1 \cdot K_2$$

alternative expression

$$\text{binding constant } K_{\text{B}} = \frac{1}{\text{dissociation constant } K_{\text{D}}}$$

Definition of the degree of binding ν

$$\nu = \frac{[\text{bound ligand } P]}{[\text{macromolecule } D]}$$

degree of binding ν

$$\nu_1 = \frac{DP}{D_{\text{free}} + DP}$$

ν for one binding site

$$\nu_2 = \frac{DP + 2 \times DP_2}{D_{\text{free}} + DP + DP_2}$$

ν for two binding sites

$$\nu = \frac{\sum_{i=1}^{n} i \cdot \frac{1}{K_i} \cdot D_{\text{frei}} \cdot P_{\text{frei}}^{i}}{\sum_{i=0}^{n} \frac{1}{K_i} \cdot D_{\text{frei}} \cdot P_{\text{frei}}^{i}} = \frac{\sum_{i=1}^{n} i \cdot \frac{1}{K_i} \cdot P_{\text{frei}}^{i}}{\sum_{i=0}^{n} \frac{1}{K_i} \cdot P_{\text{frei}}^{i}} \qquad \text{mit } K_0 = 1$$

ν for *n* binding sites (Adair equation)

Binding to a single binding site: Deriving an expression for the degree of binding ν or the fraction saturation θ

$$D_{\text{free}} + P_{\text{free}} \rightleftarrows DP \qquad K_D = \frac{D_{\text{free}} \cdot P_{\text{free}}}{DP}$$

from the Adair equation we obtain:

$$\nu_1 = \theta = \frac{\frac{1}{K_D} \cdot P_{\text{free}}}{1 + \frac{1}{K_D} \cdot P_{\text{free}}} \quad \Leftrightarrow \quad \nu_1 = \theta = \frac{P_{\text{free}}}{K_D + P_{\text{free}}}$$

Often the concentration P_{free} can not be determined but the total concentration of added protein P_{tot} is known.

$$P_{\text{free}} = P_{\text{tot}} - \nu_1 \cdot D_{\text{tot}}$$

$$\nu_1 = \frac{D_{\text{tot}} + P_{\text{tot}} + K_D - \sqrt{\left(D_{\text{tot}} + P_{\text{tot}} + K_D\right)^2 - 4 \cdot D_{\text{tot}} \cdot P_{\text{tot}}}}{2 \cdot D_{\text{tot}}}$$

Expression degree of binding ν for four sites

$$DP_1 <-> D + P; \quad K_1 = D \cdot P / DP_1; \quad DP_1 = D \cdot P / K_1$$
$$DP_2 <-> D + 2P; \quad K_2 = D \cdot P^2 / DP_2; \quad DP_2 = D \cdot P^2 / K_2$$

$$v_2 = \frac{ligand_{bound}}{macromolecule} = \frac{DP_1 + 2DP_2}{D + DP_1 + DP_2}$$

$$v_2 = \frac{\frac{1}{K_1} \cdot P_{free}^1 + \frac{2}{K_2} \cdot P_{free}^2}{1 + \frac{1}{K_1} \cdot P_{free}^1 + \frac{2}{K_2} \cdot P_{free}^2}$$

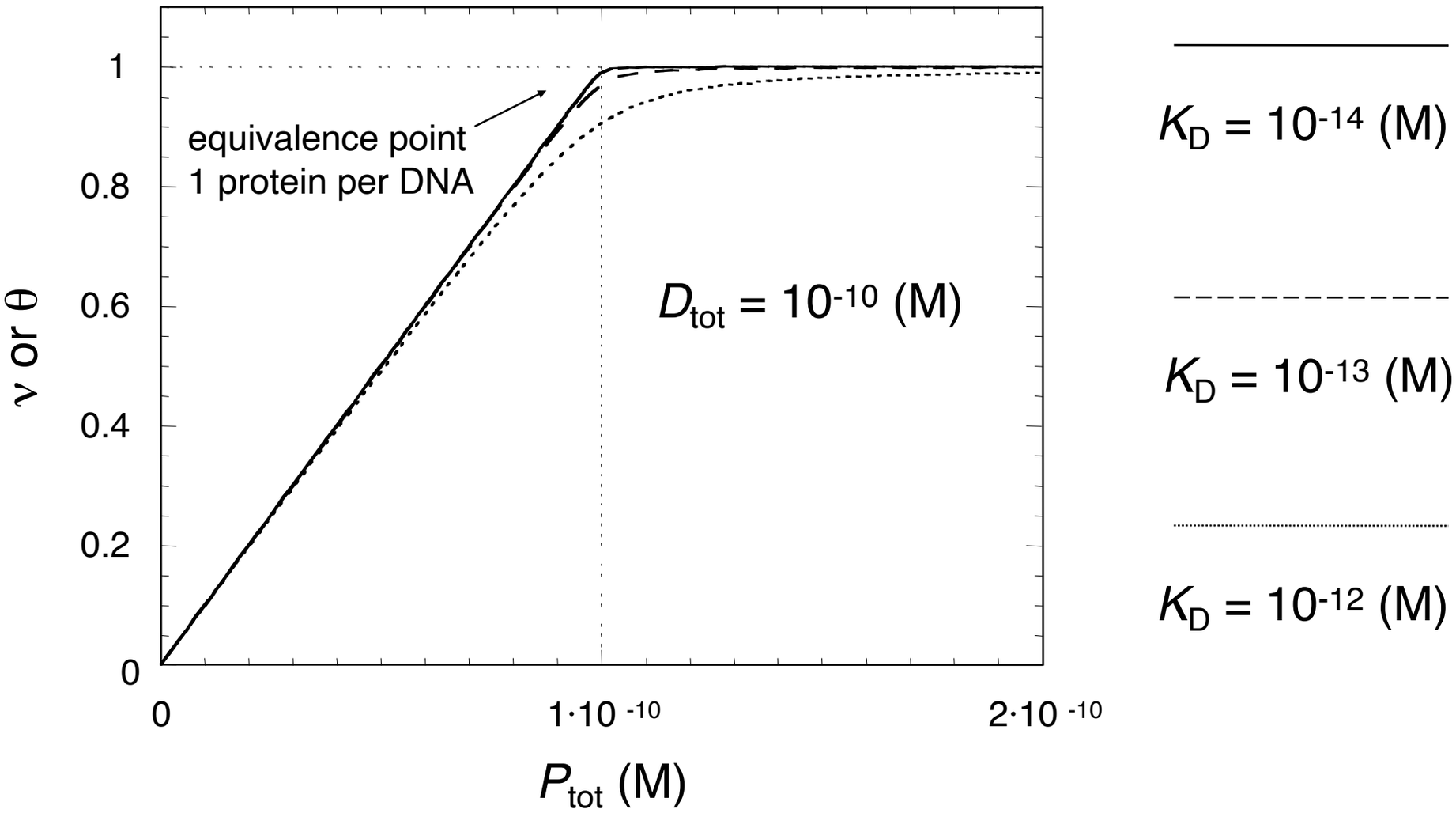
$$v_2 = \frac{K_2 \cdot P_{free}^1 + 2K_1 \cdot P_{free}^2}{K_1 \cdot K_2 + K_2 \cdot P_{free}^1 + K_1 \cdot P_{free}^2}$$

Stoichiometric titration to determine the number of binding sites

$$\frac{\nu}{n} = \theta$$

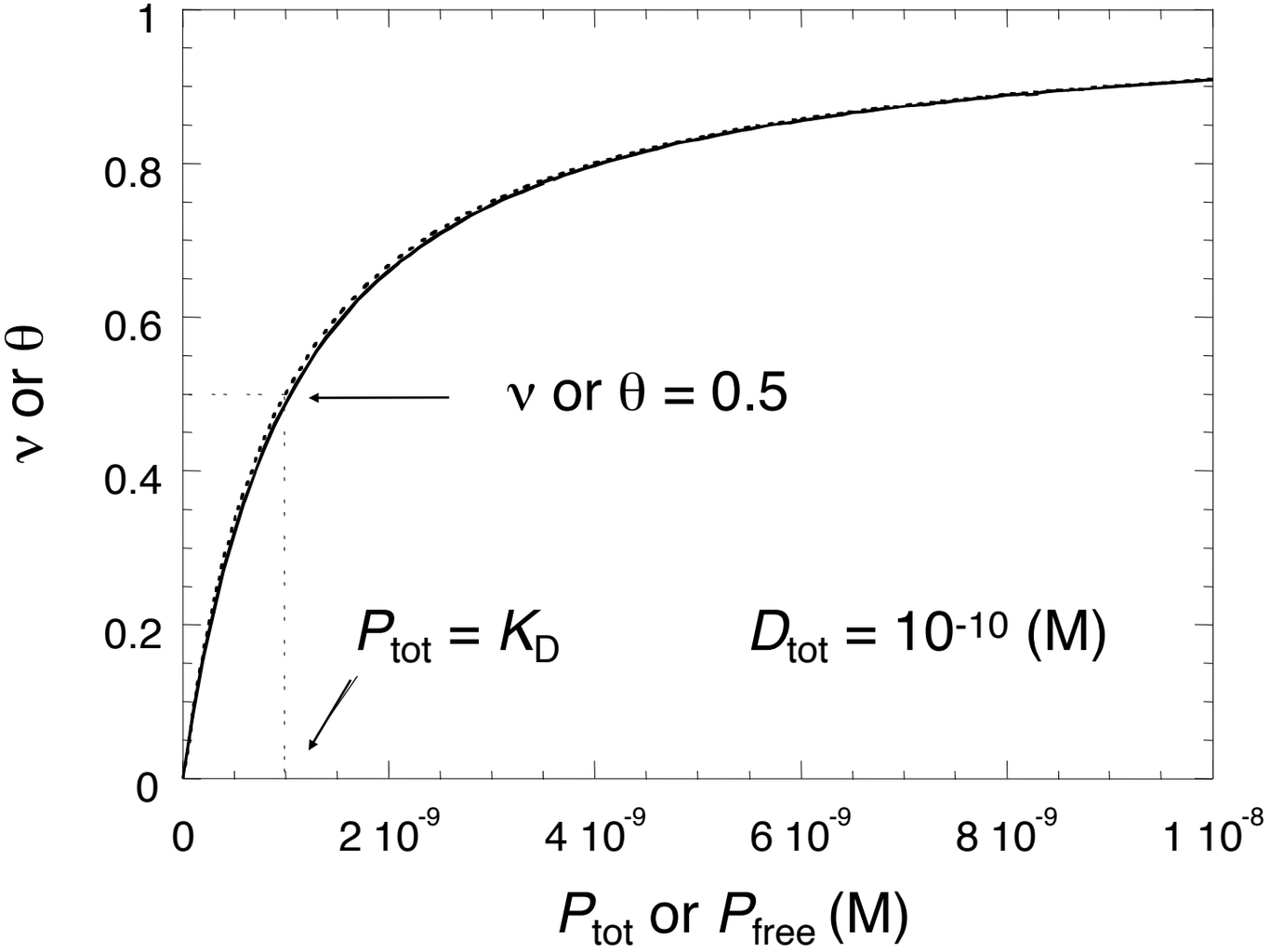
for $n = 1$

$$\nu = \theta$$



To a solution of DNA strands with a single binding site small amounts of protein *P* are added. Since the binding affinity of the protein is high (low K_D value as compared to the total DNA concentration) practically every protein binds as long as there are free binding sites on the DNA. This is termed “stoichiometric binding” or a “stoichiometric titration”.

Binding to a single binding site. Titration of DNA with a protein for the determination of the dissociation constant K_D



$K_D = 10^{-9}$ (M) $\quad \nu_1 = \dfrac{P_{free}}{P_{free} + K_D}$

$K_D = 10^{-9}$ (M) $\quad \nu_1 = \dfrac{P_{tot}}{P_{tot} + K_D}$

$$\nu_1 = \frac{P_{free}}{P_{free} + K_D} \approx \frac{P_{tot}}{P_{tot} + K_D} \quad \text{if} \quad P_{free} \approx P_{tot} \quad \text{d. h.} \quad 10 \times D_{tot} \leq K_D$$

Dissociation constant for binding of a single ligand to its target

1. K_D is a concentration and has units of mol per liter

2. K_D gives the concentration of ligand that saturates 50% of the sites if total binding site concentration is ≤ 0.1 x K_D and free ligand total equals total ligand concentrations. -> titration to measure K_D

3. If the ligand concentration is ≥ 10 x K_D almost all binding sites are saturated. -> “stoichiometric” titration to determine binding sites

4. Adair equation can be used to derive an equation that relates saturation of macromolecule (q, n) with K_D for binding of *n* ligands to a macromolecule with K_1, K_2 … K_n