L5 Enzyme functional nature: Activity measurement

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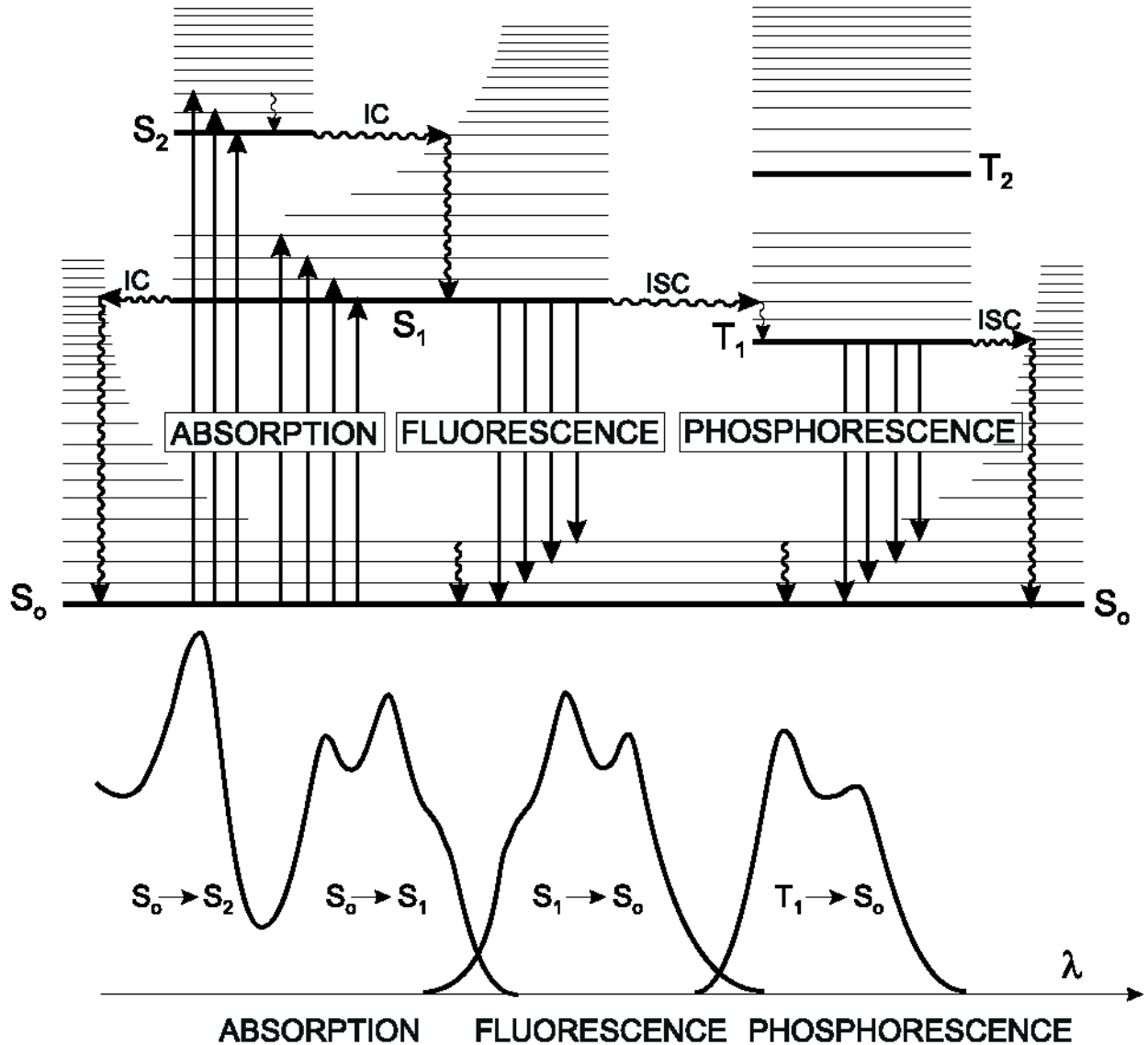
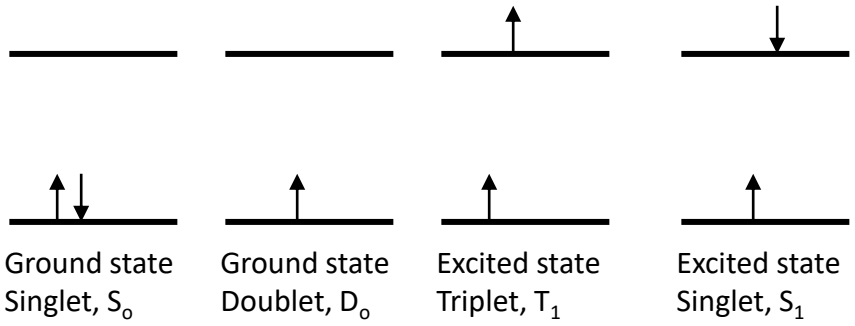
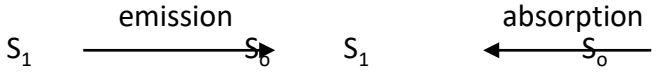
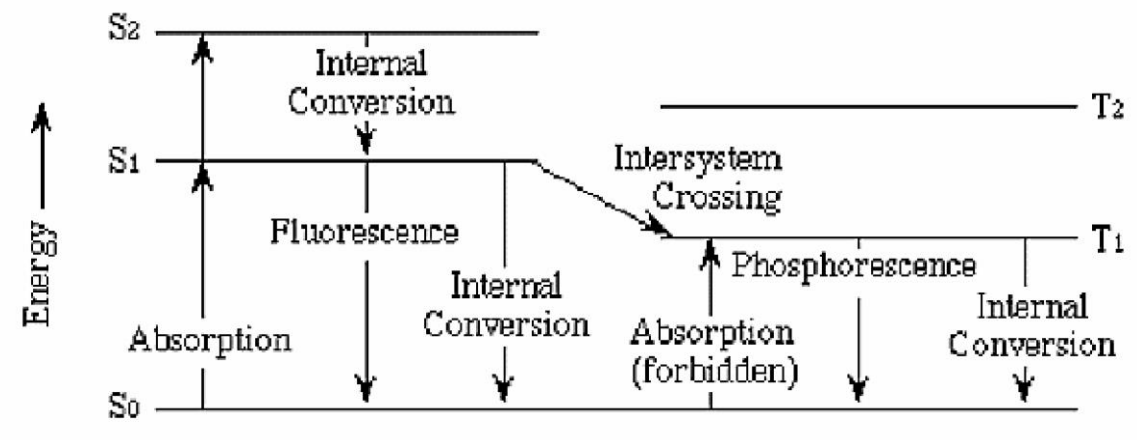
Perrin-Jablonski diagram





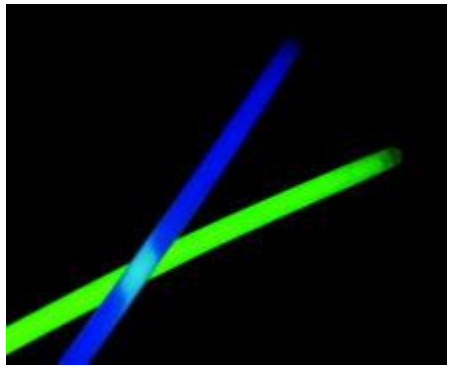




S is singlet and T is triplet.

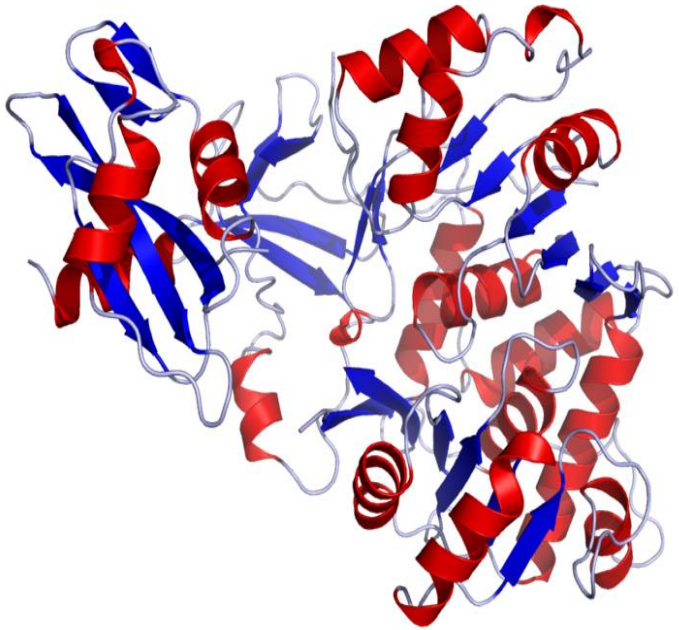
The S_0 state is the ground state and the subscript numbers identify individual states.

Chemi-luminenscence



[**A**] + [**B**] → [◊] → [*Products*] + light

luminol + hydrogen peroxide → 3-APA[◊] → 3-APA + light



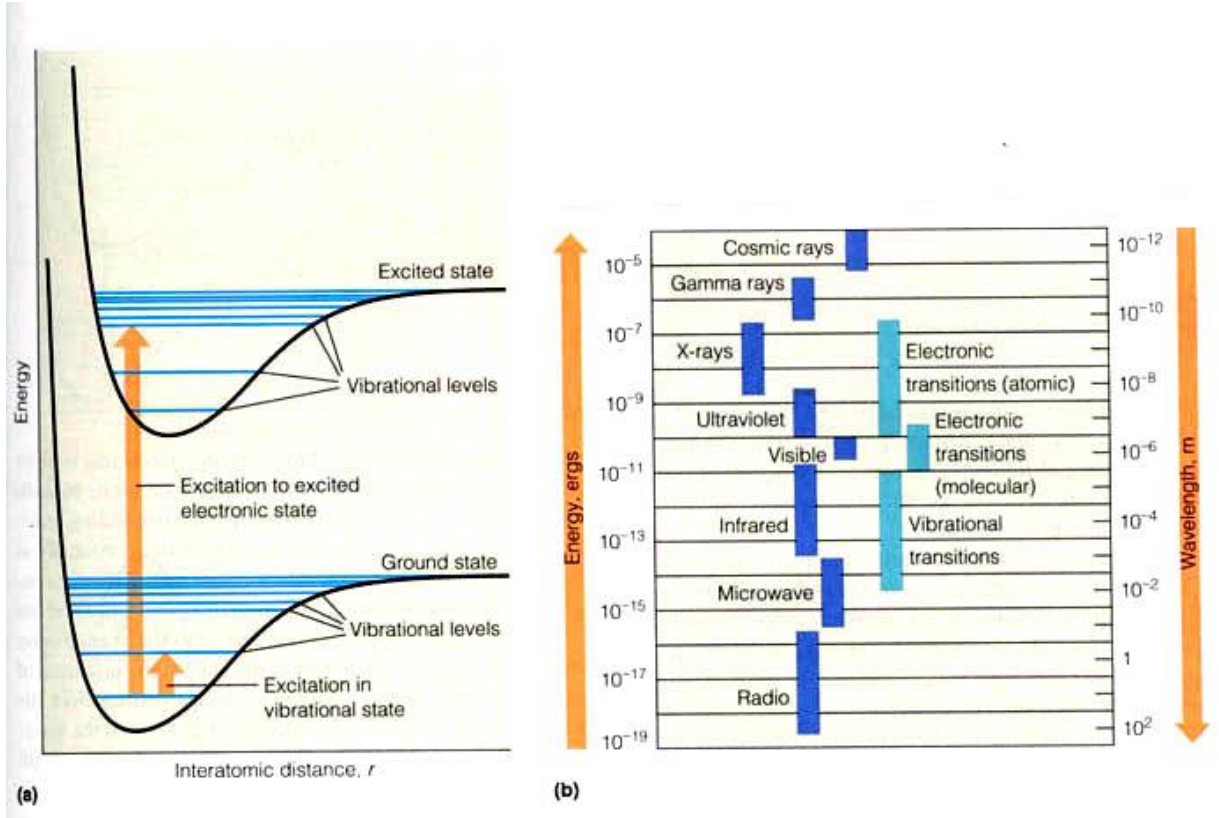
luciferin + ATP + Mg^{2+}

⇩ **luciferase**

oxyluciferin + AMP + CO_2

+

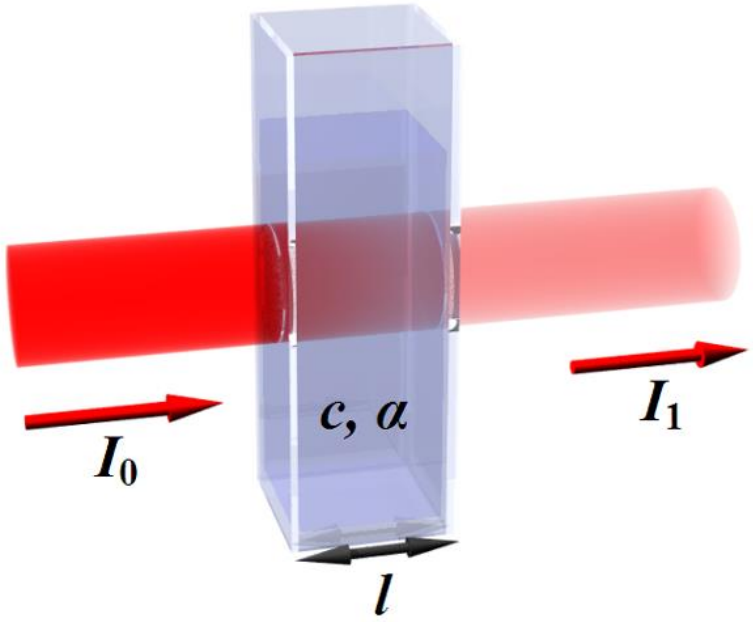
light



Frank-Condon Principle

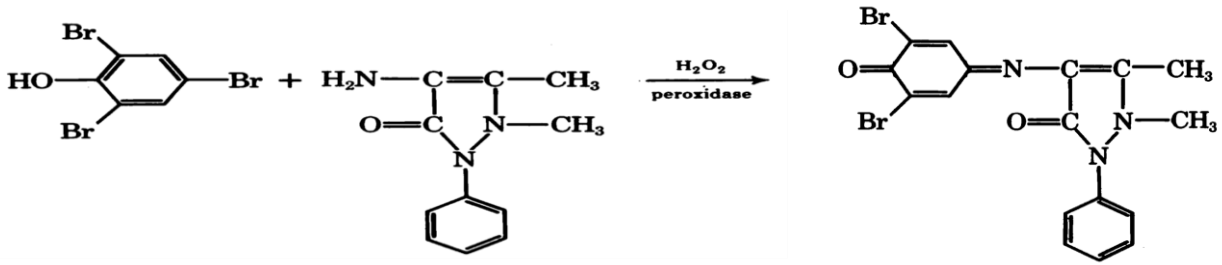
- The nuclear motion (10^{-13} s) is negligible during the time required for an electronic excitation (10^{-16} s).
- Since the nuclei do not move during the excitation, the internuclear distances remain constant and “the most probable component of an electronic transition involves only the vertical transitions”.

Absorption



$$I_1/I_0 = e^{-\alpha lc}$$

l is the pass length
C is the concentration of absorbing material
α is the absorption coefficient



Color reaction

Absorption: The Beer-Lambert Law

- The Beer-Lambert law sortof has the wrong name…

Pierre Bouguer
(1698-1758)
Astronomer: Light is diminished as it passes through the atmosphere.

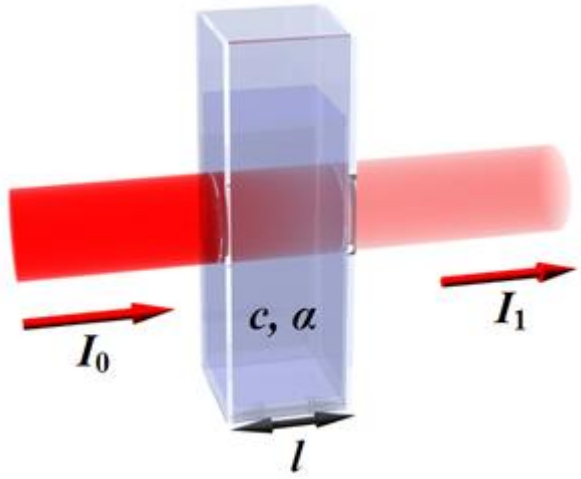
Johan Lambert
(1728-1777)
Mathematician, first to prove that π is irrational. No absorption coefficient.

$$A = -\log(I_1 / I_0) = \varepsilon c l$$

Extinction coefficient

Concentration

Path length



August Beer (1825-1863): Added absorption co-efficient and related to conc. in solution.

Assumptions

TABLE 3-1
Assumptions of the absorption law

1. The incident radiation is monochromatic.
2. The absorbers (molecules, atoms, ions, etc.) act independently of each other.
3. The incident radiation consists of parallel rays, perpendicular to the surface of the absorbing medium.
4. The pathlength traversed is uniform over the cross section of the beam. (All rays traverse an equal distance of the absorbing medium.)
5. The absorbing medium is homogeneous and does not scatter the radiation.
6. The incident flux is not large enough to cause saturation effects. (Lasers can cause such effects, as discussed in Chapters 11 and 15.)

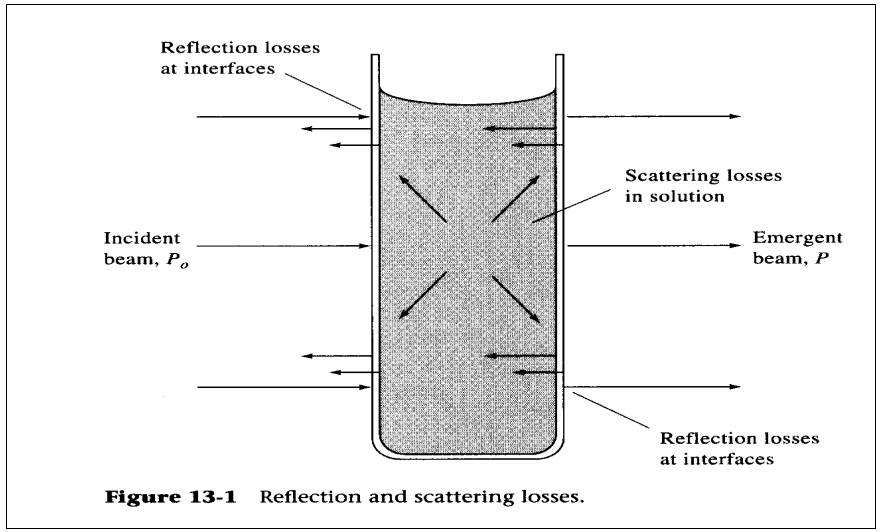
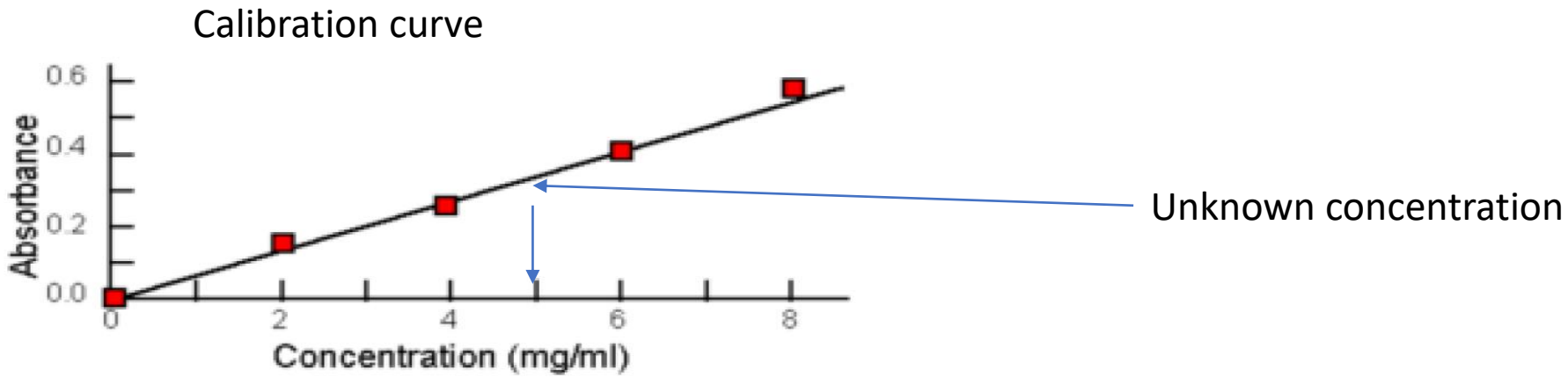


Figure 13-1 Reflection and scattering losses.

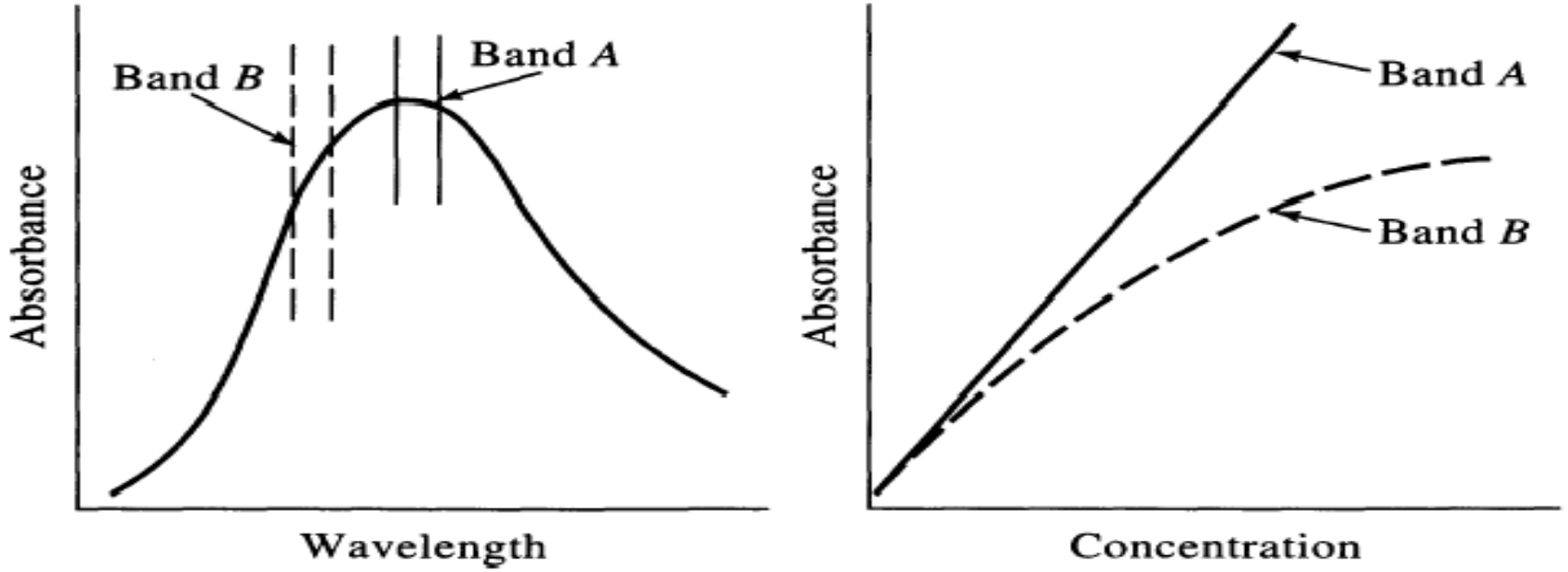
Beer's law and mixtures

- Each analyte present in the solution absorbs light!
- The magnitude of the absorption depends on its ε
- $A_{total} = A_1 + A_2 + \ldots + A_n$
- $A_{total} = \varepsilon_1 b c_1 + \varepsilon_2 b c_2 + \ldots + \varepsilon_n b c_n$
- If $\varepsilon_1 = \varepsilon_2 = \varepsilon_n$ then simultaneous determination is impossible
- Need nλ's where ε's are different to solve the mixture



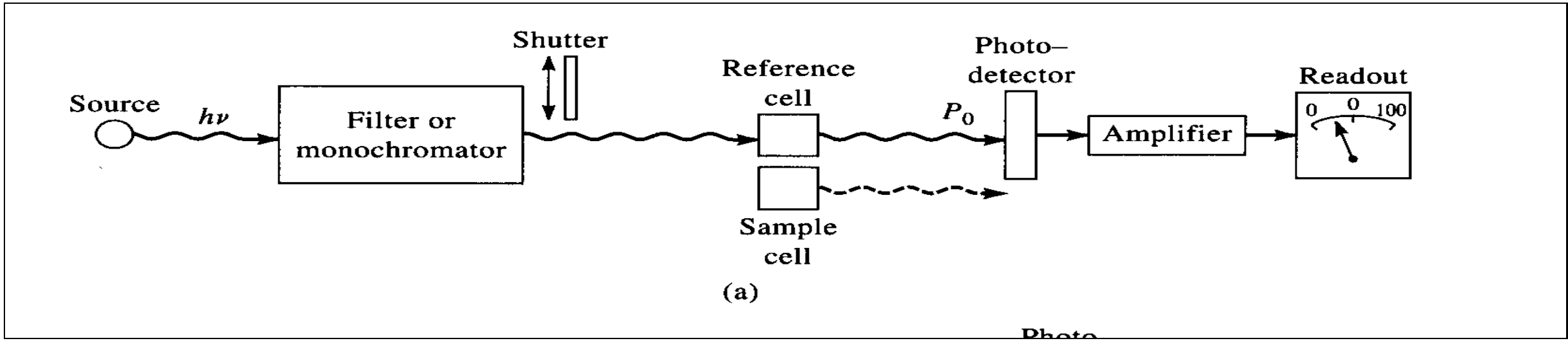
Beer's Law Limitation

- Polychromatic Light
  - More than one wavelength cannot be used



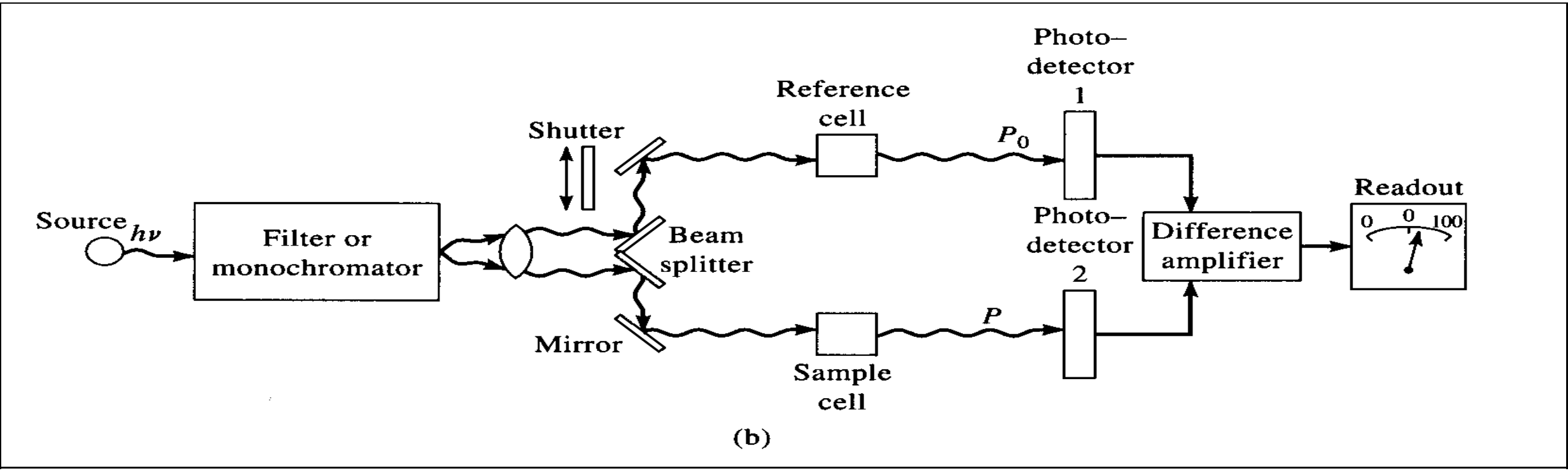
Ideally, a monochromator will pass radiation of a single wavelength, but in reality the monochromator passes a band of radiation. The bandwidth of the spectrometer will affect the linearity of Beer's Law.

General Instrument Designs
Single beam



- 0% T is set with shutter in the beam path.
- 100% T is set with a reference in the beam path.
- Measurement is then made with the sample in the beam path.

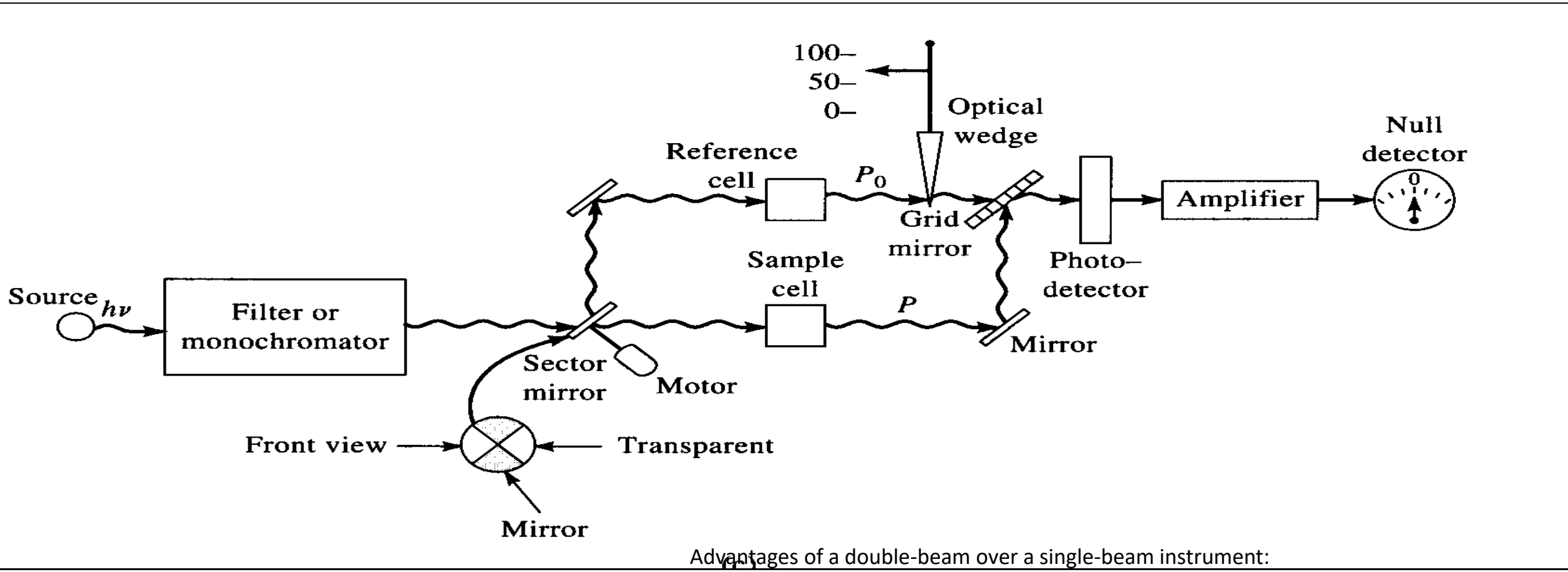
General Instrument Designs
Double Beam: Space resolved



Sample and reference are measured simultaneously and the signal from the reference is subtracted from the sample signal.

A major drawback of this type of instrument is the requirement of two detectors, which makes the instrument more expensive.

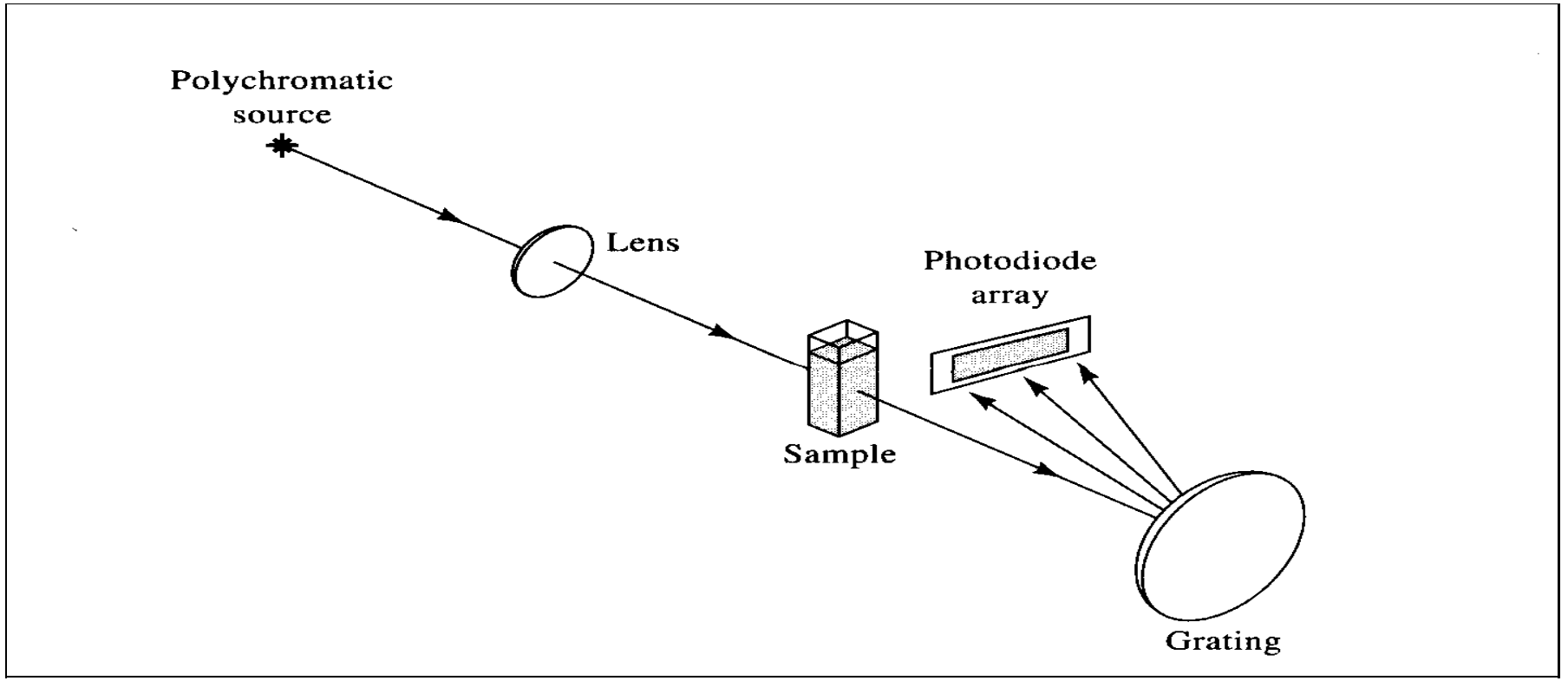
General Instrument Designs
Double Beam: Time resolved



Advantages of a double-beam over a single-beam instrument:

- **Compensate for variations in the source intensity.**
- **Compensate for drift in the detector and amplifier.**
- **Compensate for variation in intensity as a function of wavelength.**

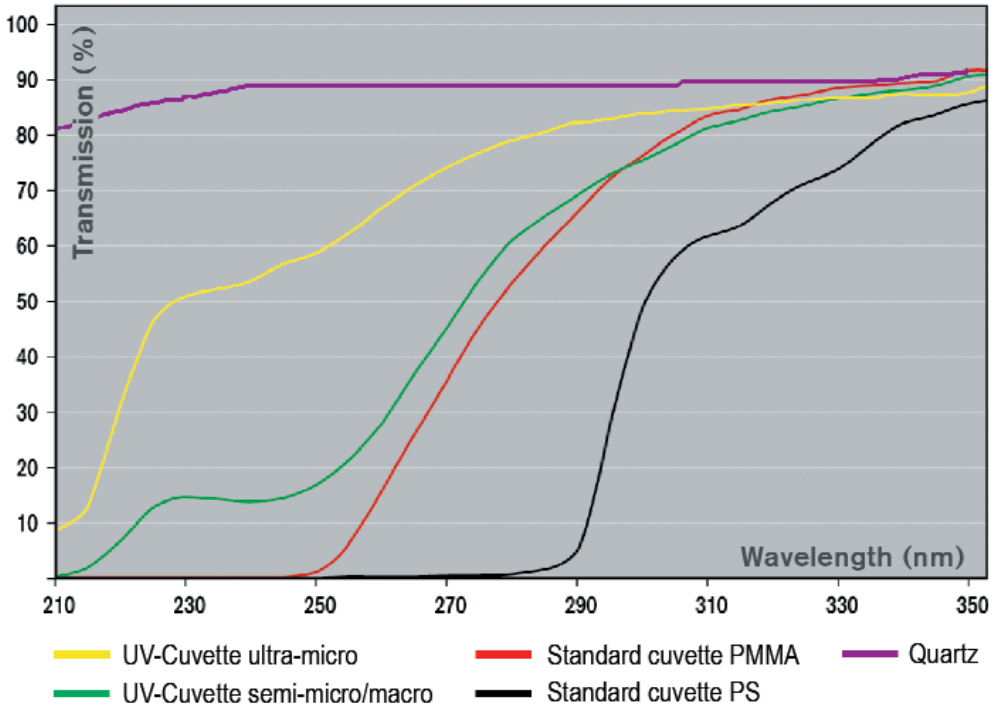
Multi-channel Design

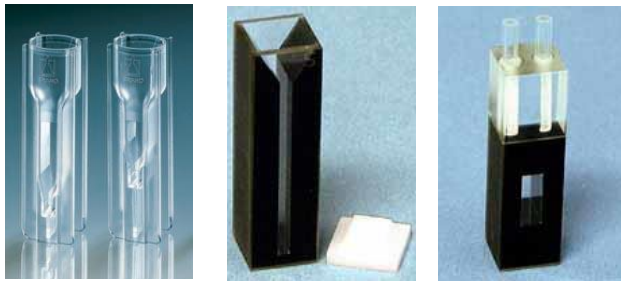


- Able to “scan” (collect) an entire spectrum in ~ 0.1 sec.
- Uses signal averaging over a period of 1 sec or more to enhance signal-to noise ratio.
- Have high throughput of radiant energy due to the minimal optics.
- Typically use a deuterium lamp source for a spectral range of 200nm🕓🔗820 nm and have a spectral bandwidth (resolution) of 2 nm.

Sample Compartments/Holders

- The sample itself is held in a cuvette, usually plastic or quartz:





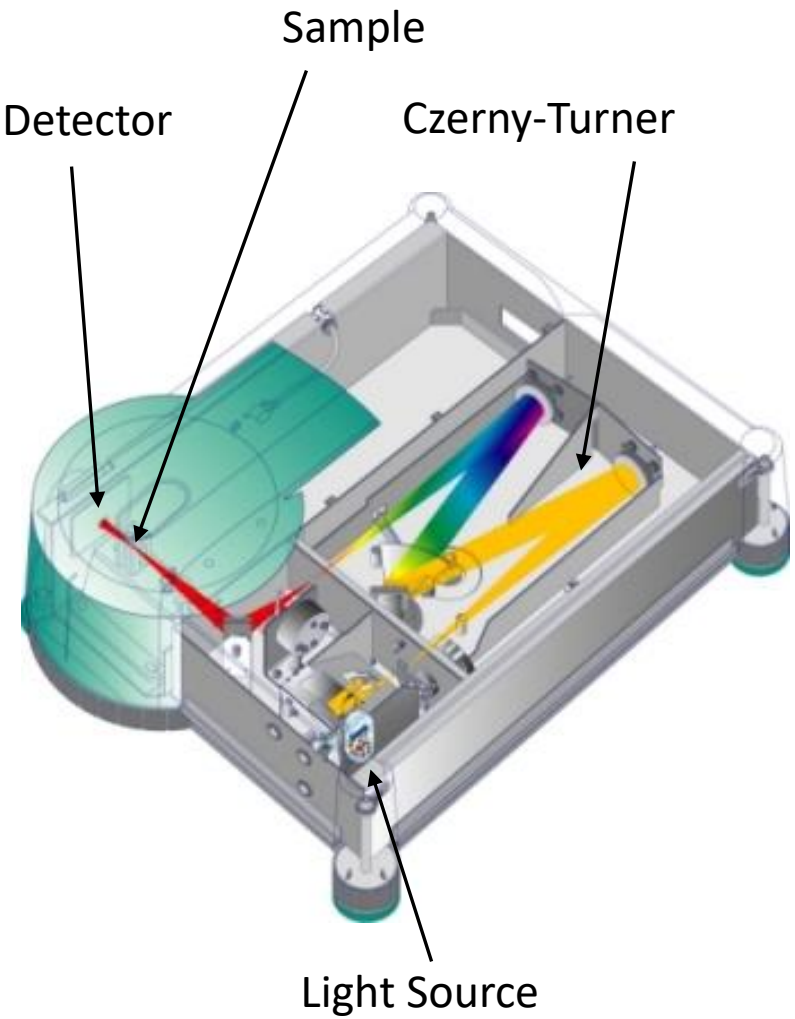

UV-1800
RAYLEIGH

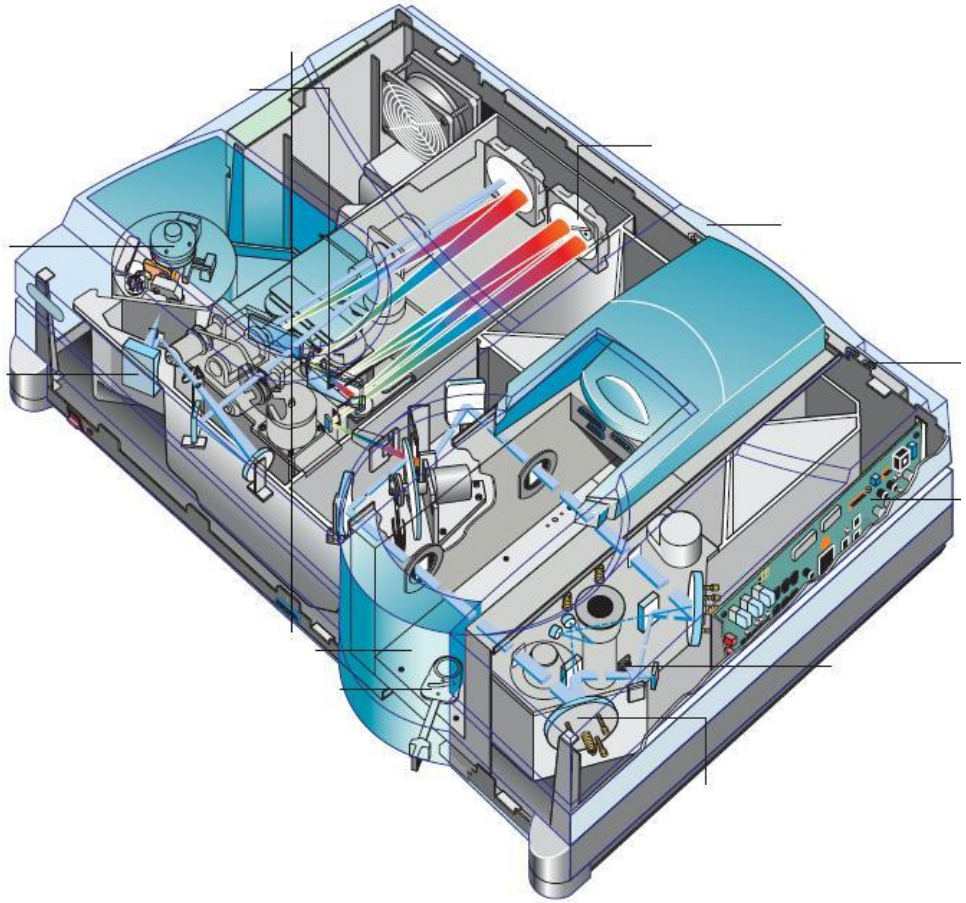

photonics
CCD Array UV-Vis Spectrophotometer


Ocean Optics
HR2000+CG


NanoDrop
ND-1000
Spectrophotometer

The Whole Instrument

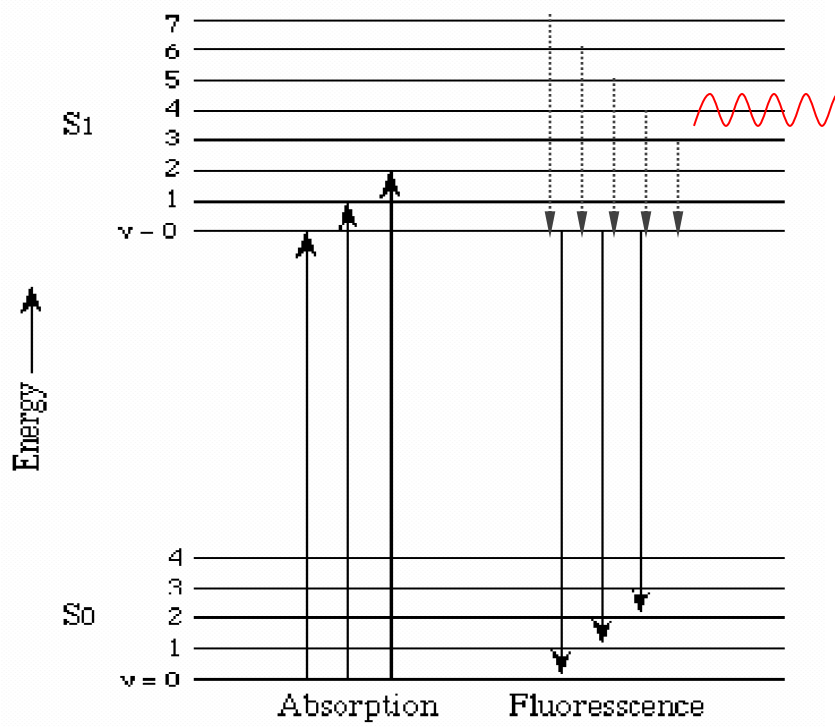


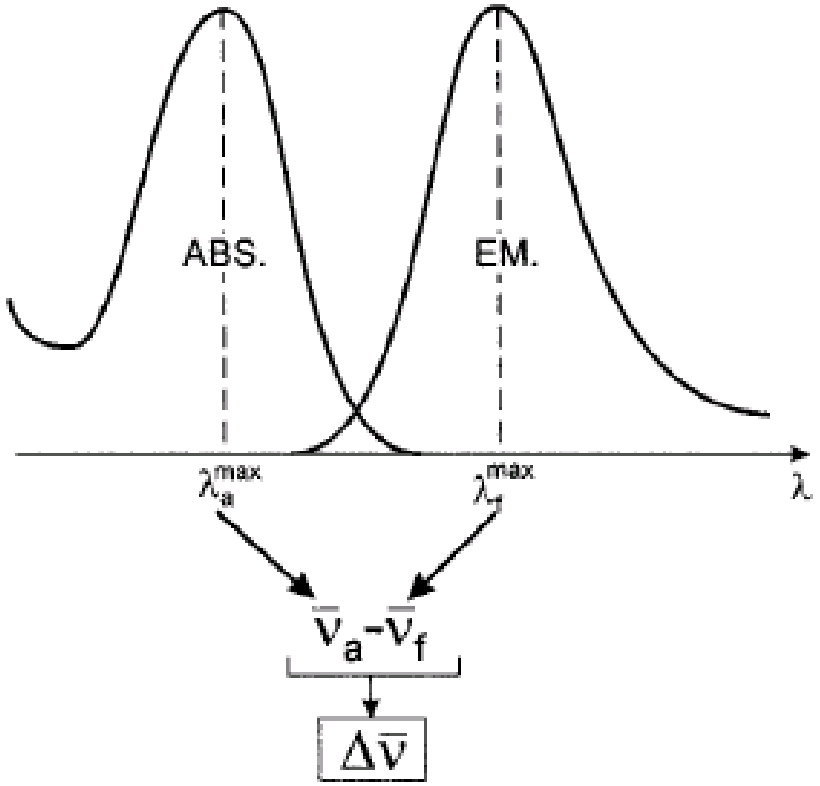


Stokes shift

The Stokes shift is the gap between the maximum of the first absorption band and the maximum of the fluorescence spectrum

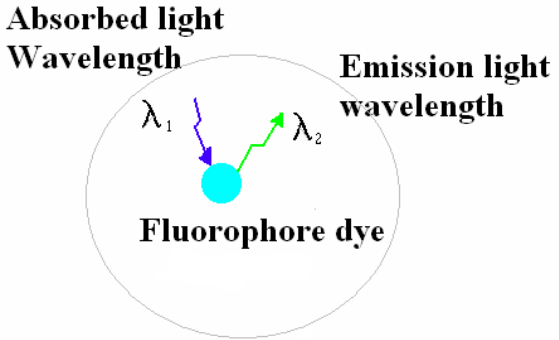
loss of vibrational energy in the excited state as heat by collision with solvent



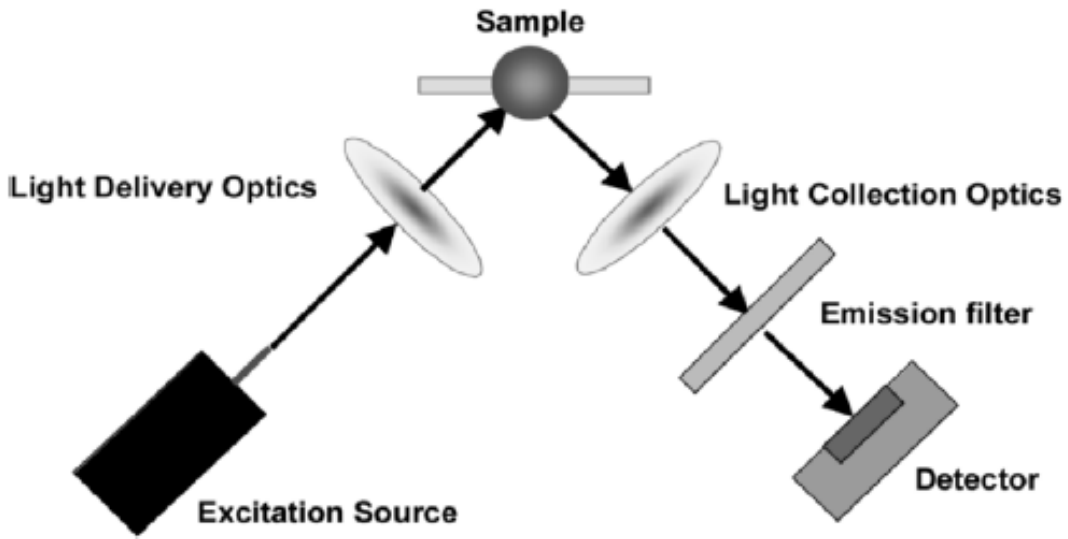


Transducers-Optical methods- Florescence

Fluorescence is a molecular absorption of light at one wavelength and its instantaneous emission of at longer wavelengths. Some molecules fluoresce naturally and others such as DNA can be modified for fluorescence detection by attachment of special fluorescent dyes

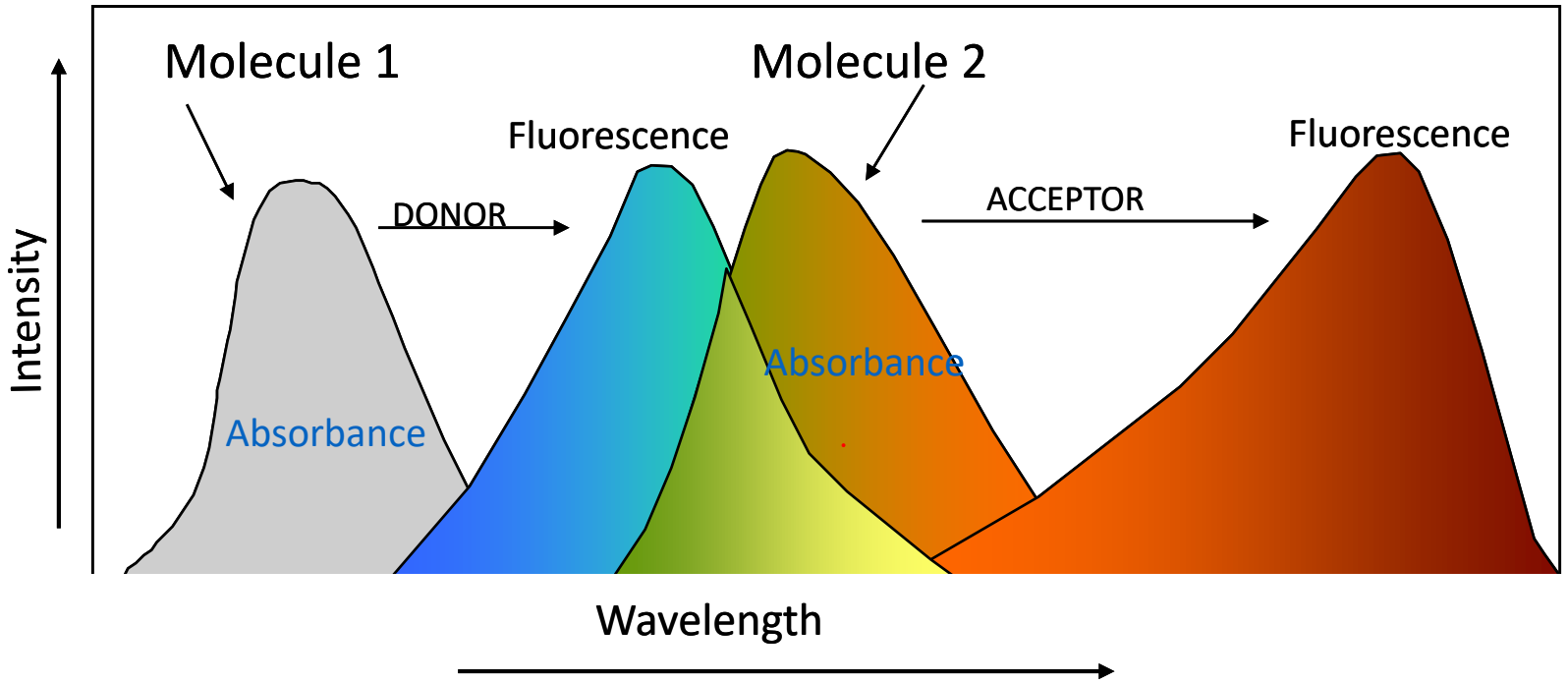


An optical system for
Florescence measurement →



I. Principles of fluorescence

- Fluorescence energy transfer (FRET)



Non radiative energy transfer – a quantum mechanical process of resonance between transition dipoles

Effective between 10-100 Å only

Emission and excitation spectrum must significantly overlap

Donor transfers non-radiatively to the acceptor

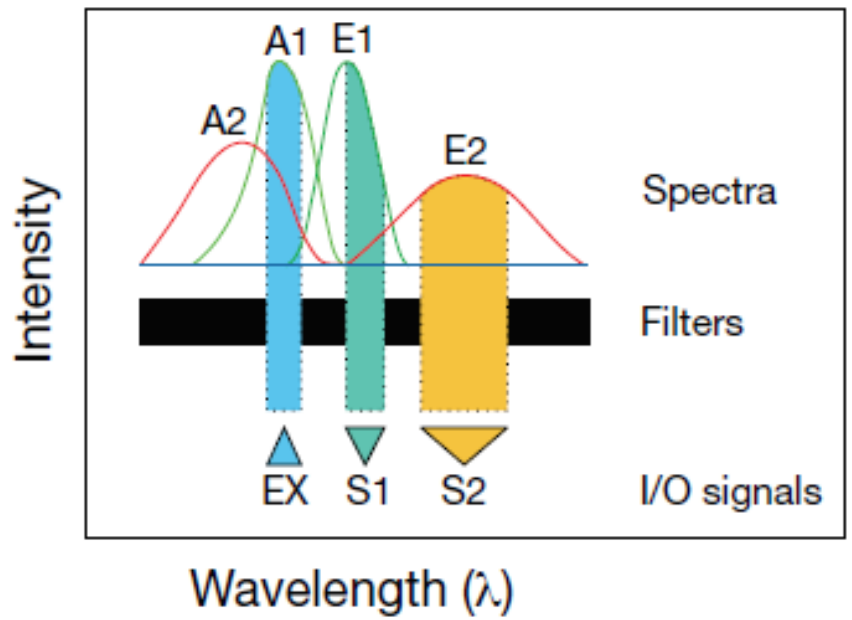
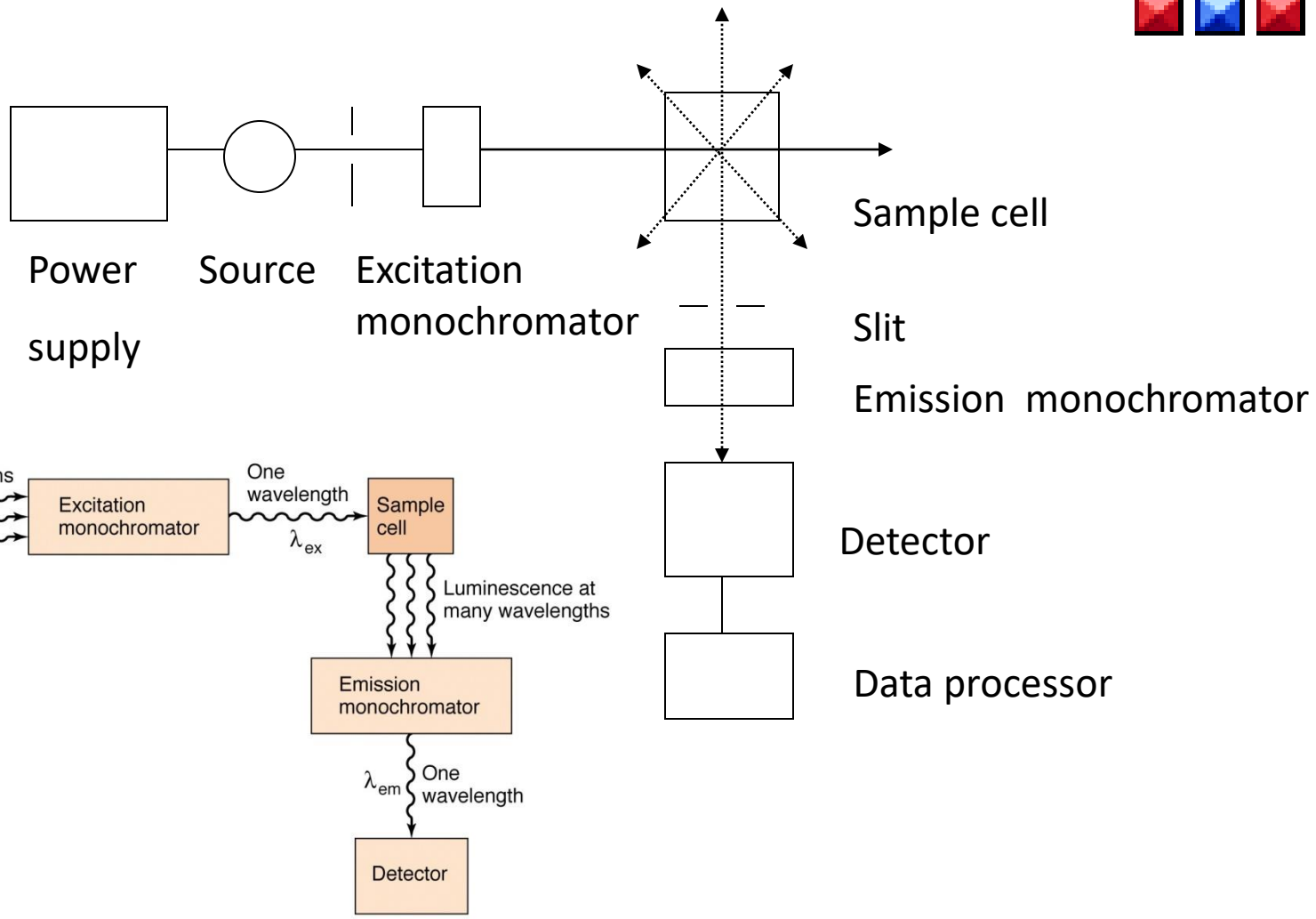
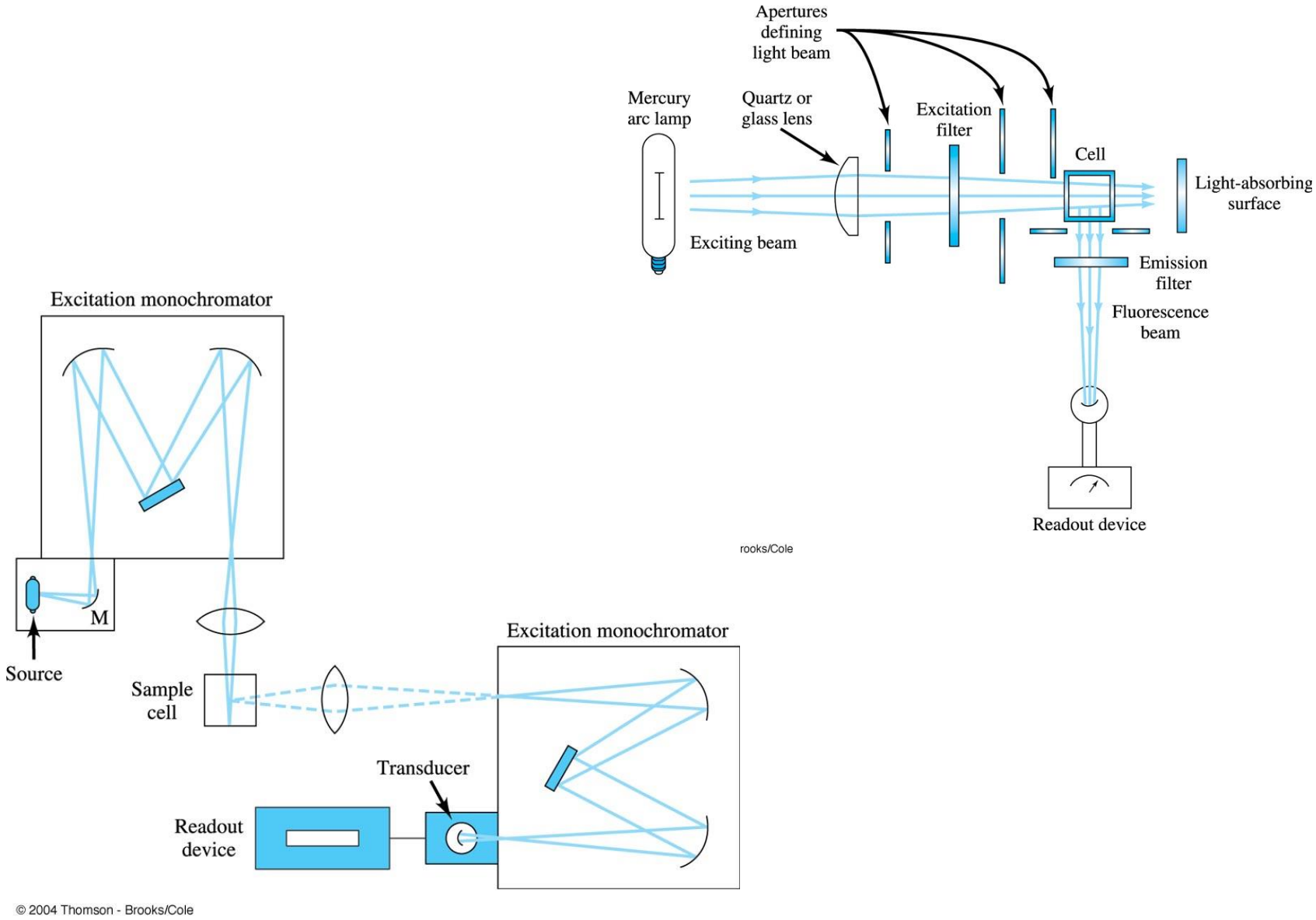


Figure 3 Fluorescence detection of mixed species. Excitation (EX) in overlapping absorption bands A1 and A2 produces two fluorescent species with spectra E1 and E2. Optical filters isolate quantitative emission signals S1 and S2.

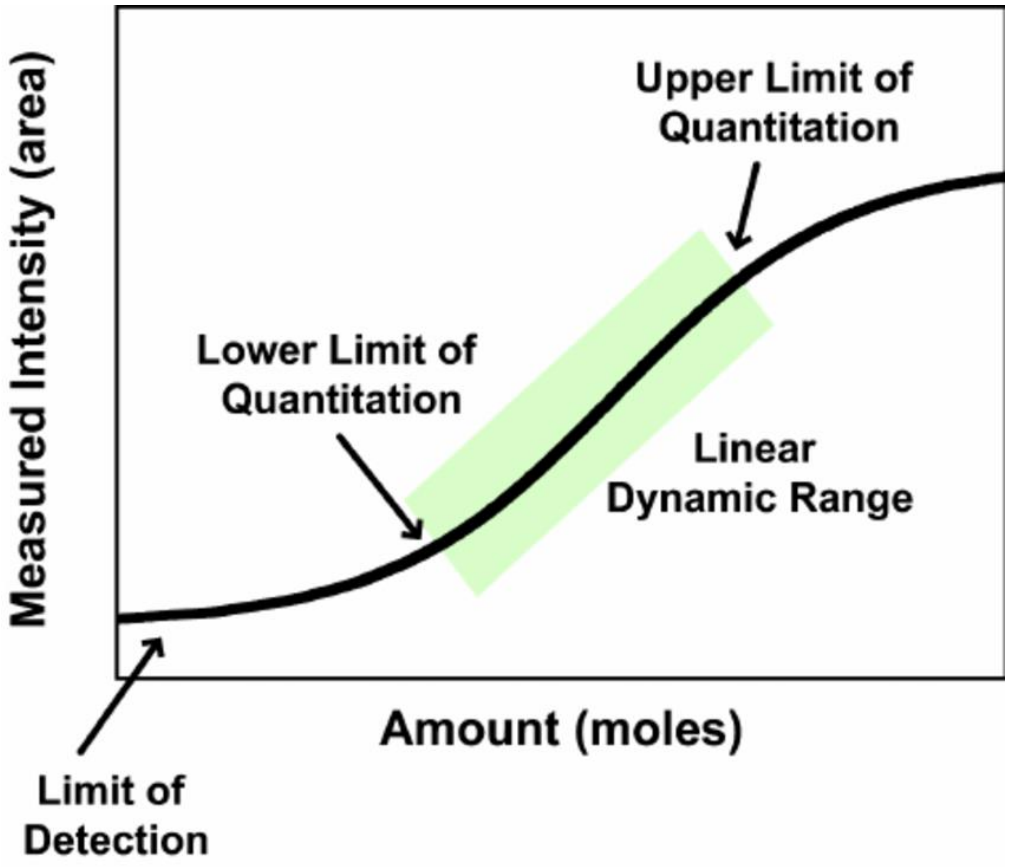
Instrumentation for fluorescence spectroscopy



General layout of fluorescence spectrophotometer



Schematic diagram of a typical spectrofluorometer.



Background noise ??

Sensitivity ?

Specificity ?

Figure 4 from Strategies and Challenges in Measuring Protein Abundance Using Stable Isotope Labeling and Tandem Mass Spectrometry; DOI: 10.5772/33421

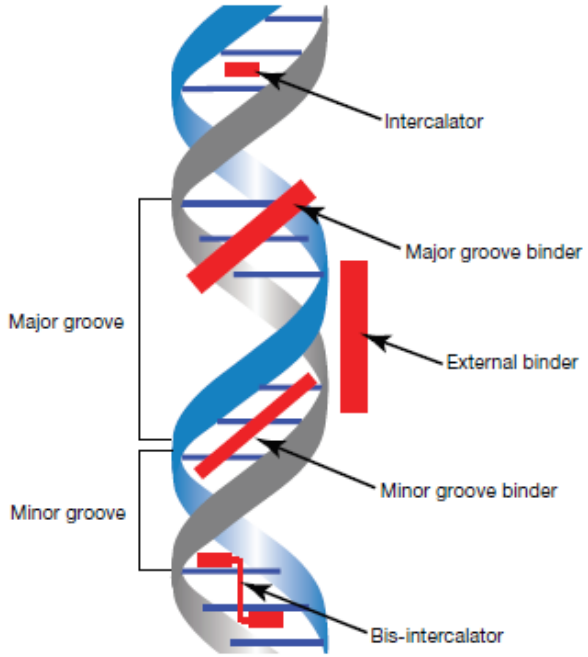


Figure 8.1.1 Schematic diagram showing the different binding modes of dyes (and other ligands) to DNA.

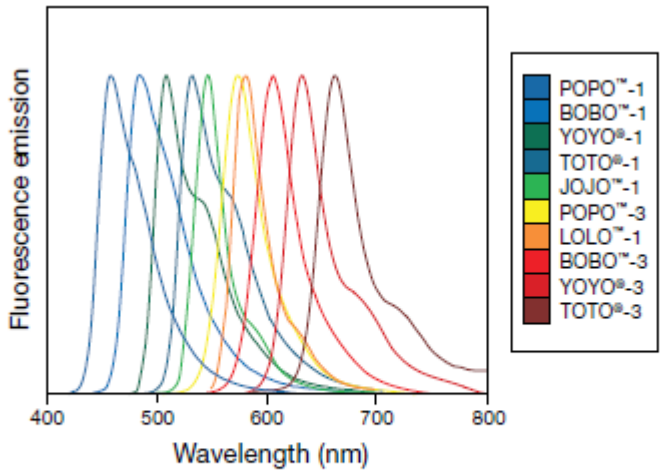
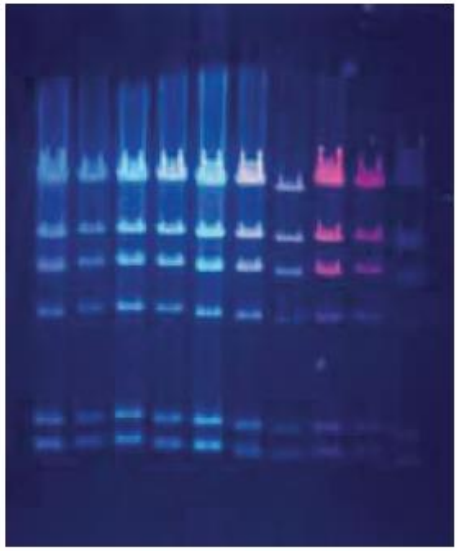


Figure 8.1.2 Normalized fluorescence emission spectra of DNA-bound cyanine dimers, identified by the color key on the sidebar.



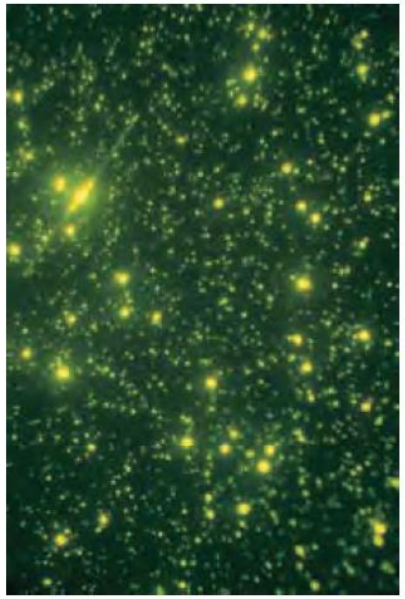
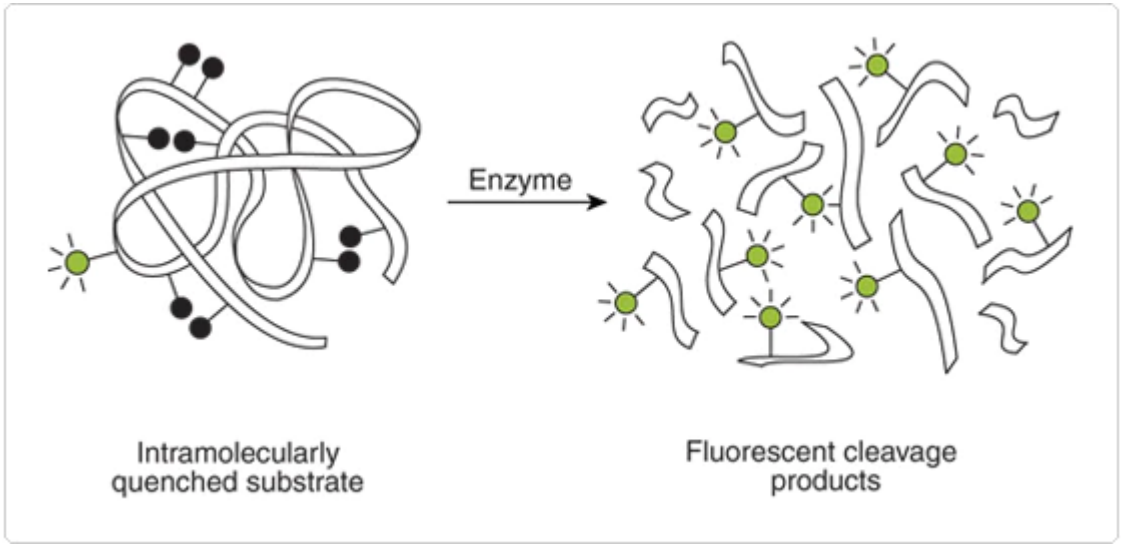


Figure 8.3.12 An environmental sample containing marine viruses (smallest dots), bacteria (larger, brighter dots) and a diatom (long thin cell with prominent nucleus) stained with SYBR® Green I nucleic acid stain (S7563, S7567, S7585). Image contributed by Jed Fuhrman, University of Southern California.

Enzyme fluorogenic substrates



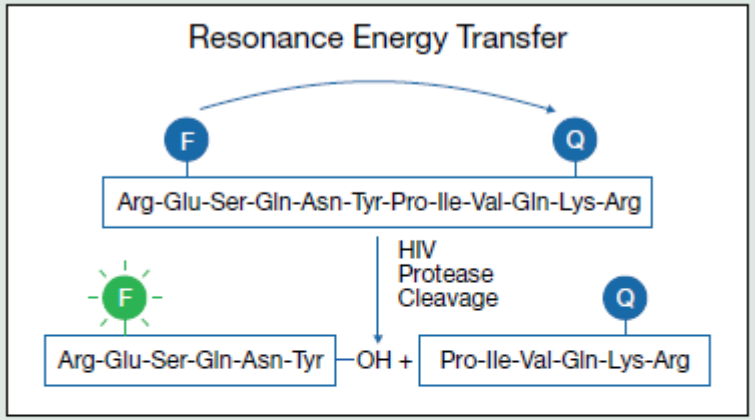


Figure 2 Principle of the fluorogenic response to protease cleavage exhibited by HIV protease substrate 1 (H2930). Quenching of the EDANS fluorophore (F) by distance-dependent resonance energy transfer to the dabcyl quencher (Q) is eliminated upon cleavage of the intervening peptide linker.

Some more applications

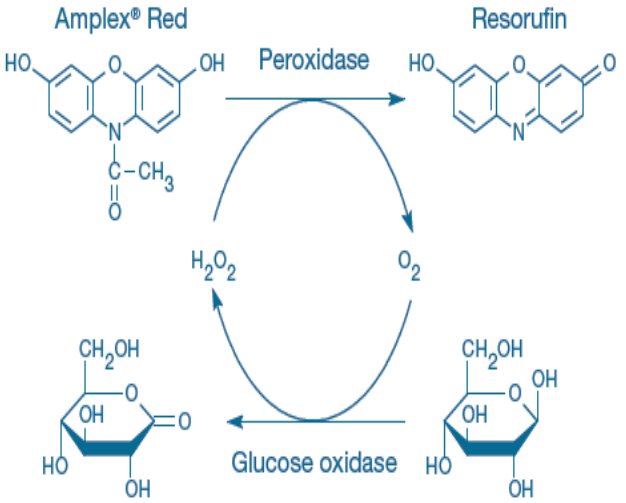


Figure 10.2.15 Principle of coupled enzymatic assays using our Amplex® Red reagent. Oxidation of glucose by glucose oxidase results in generation of H_2O_2, which is coupled to conversion of the Amplex® Red reagent to fluorescent resorufin by HRP. The detection scheme shown here is used in our Amplex® Red Glucose/Glucose Oxidase Assay Kit (A22189).

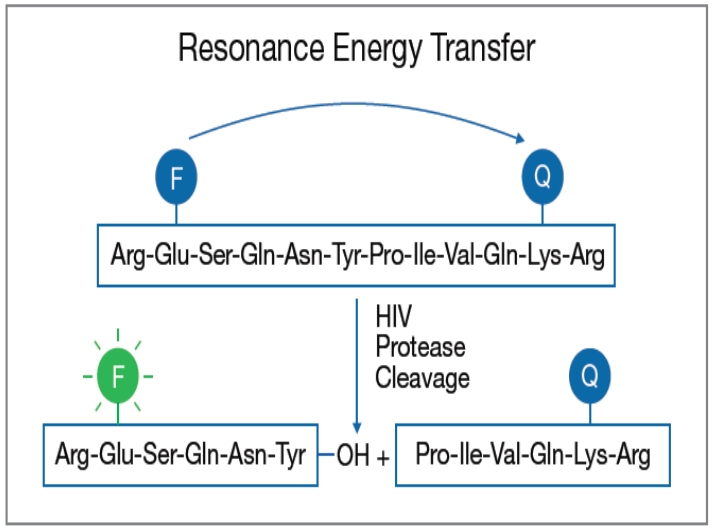
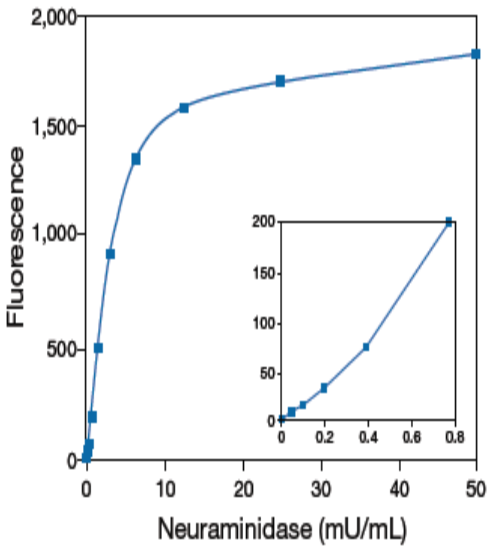
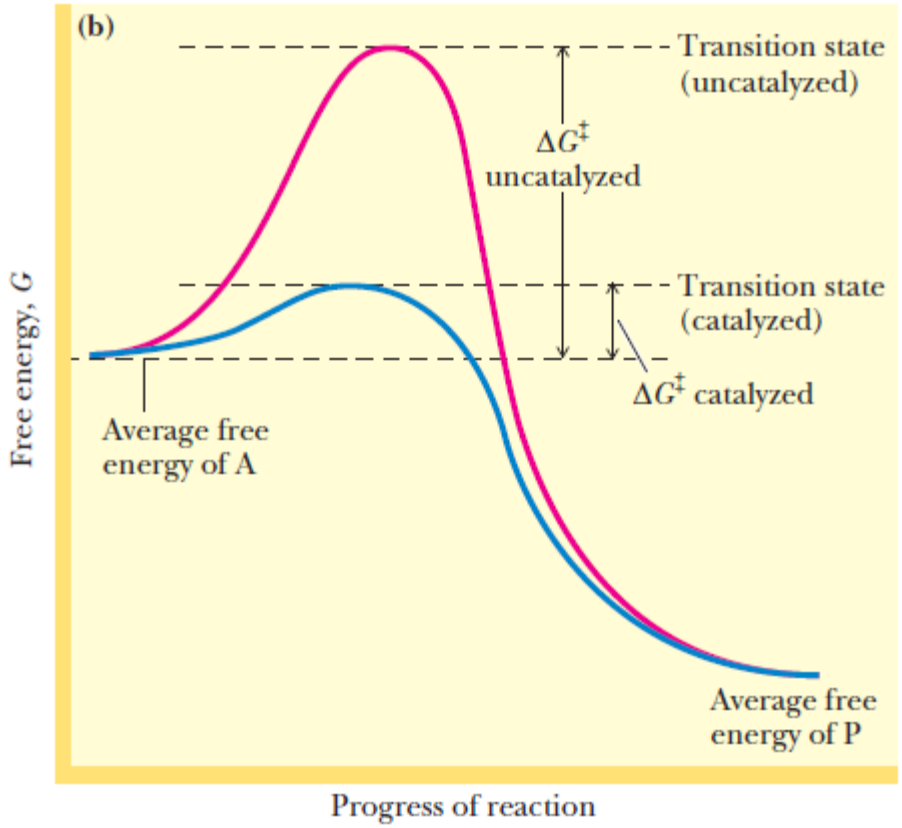


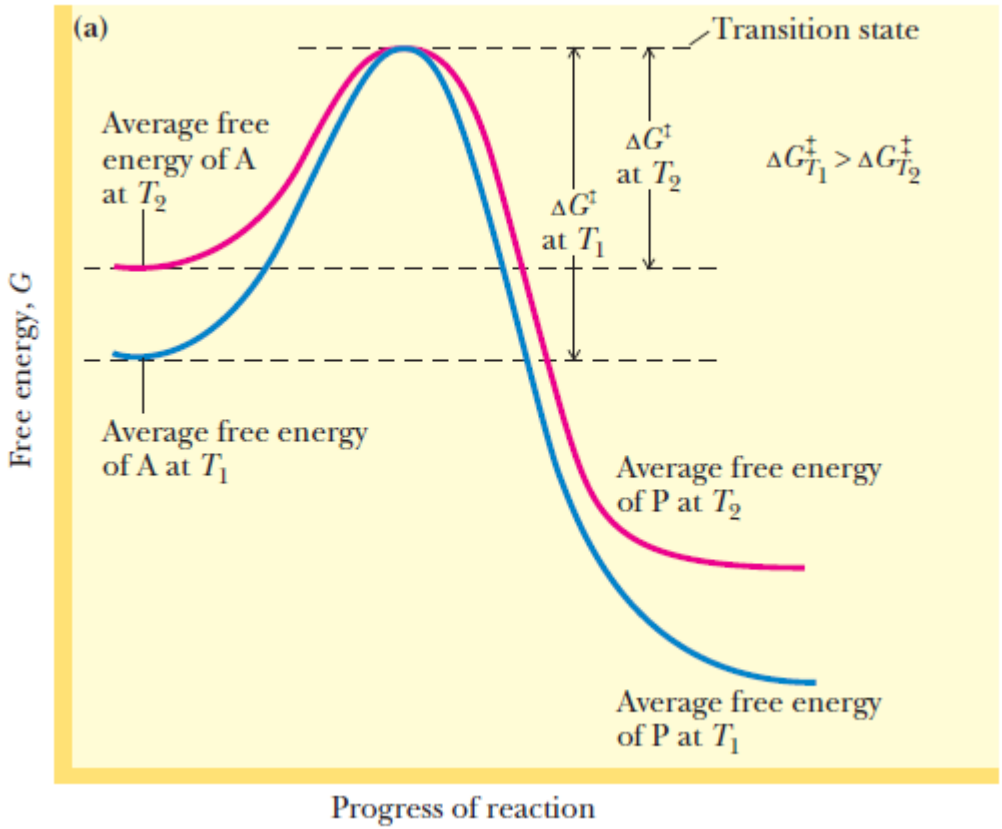
Figure 10.1.16 Principle of the fluorogenic response to protease cleavage exhibited by HIV protease substrate 1 (H2930). Quenching of the EDANS fluorophore (**F**) by distance-dependent resonance energy transfer to the dabcyl quencher (**Q**) is eliminated upon cleavage of the intervening peptide linker.



Enzyme thermodynamics

Mechanism :Catalysts Lower the Free Energy of Activation for a Reaction





Gibbs free energy

- Thermodynamics: changes in free energy, entropy, …

$$\Delta G = \Delta H - T \cdot \Delta S$$
$$\Delta G = (\Delta U + P \cdot \Delta V) - T \cdot \Delta S$$

- For nearly all biochemical reactions ΔV is small and ΔH is almost equal to ΔU

- Hence, we can write:

$$\Delta G = \Delta U - T \cdot \Delta S$$

The Enthalpic term

- Changes in bonding
  - van der Waals
  - Hydrogen bonding
  - Charge interactions

The Entropic term

- Changes the arrangement of the solvent or counterions
- Reflects the degrees of freedom
- Rotational & Translational changes

If ΔG is negative

Energy was released, products are simpler, greater entropy (2nd Law of Thermodynamics)

Exergonic / exothermic reaction (spontaneous)

If ΔG is positive

Energy input, product more complex, energy needed to go against 2nd Law

Endergonic / endothermic (non-spontaneous)

Protein folding

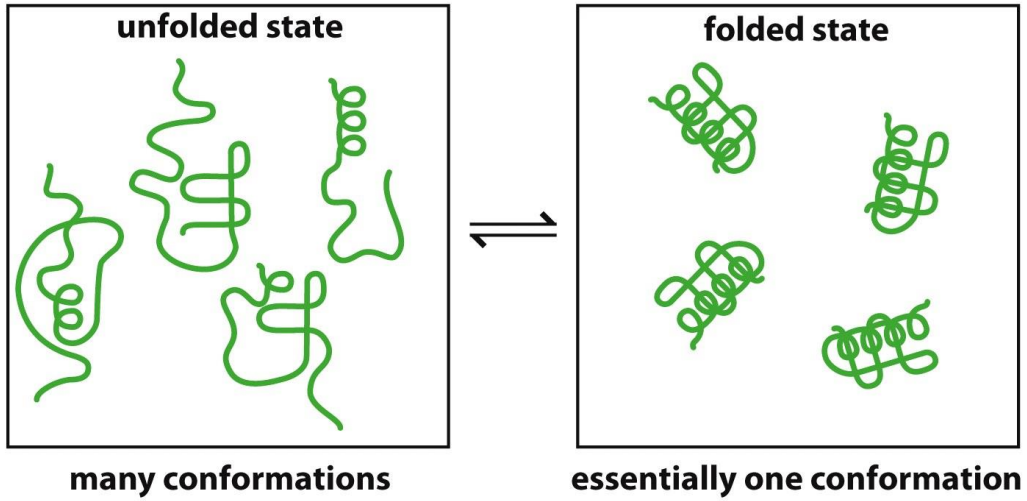


Figure 10.13 The Molecules of Life (© Garland Science 2013)

Protein folding

(U) Unfolded ⇌ Folded (F)

$K_{Folding} = \frac{(F)}{(U)} = \frac{1}{K_{unfolding}}$

$\Delta G^{\circ}_{unfolding} = \Delta H^{\circ} - T\,\Delta S^{\circ}$

Protein folding is a spontaneous process?

$\Delta G < 0$???

Enthalpy change in protein folding

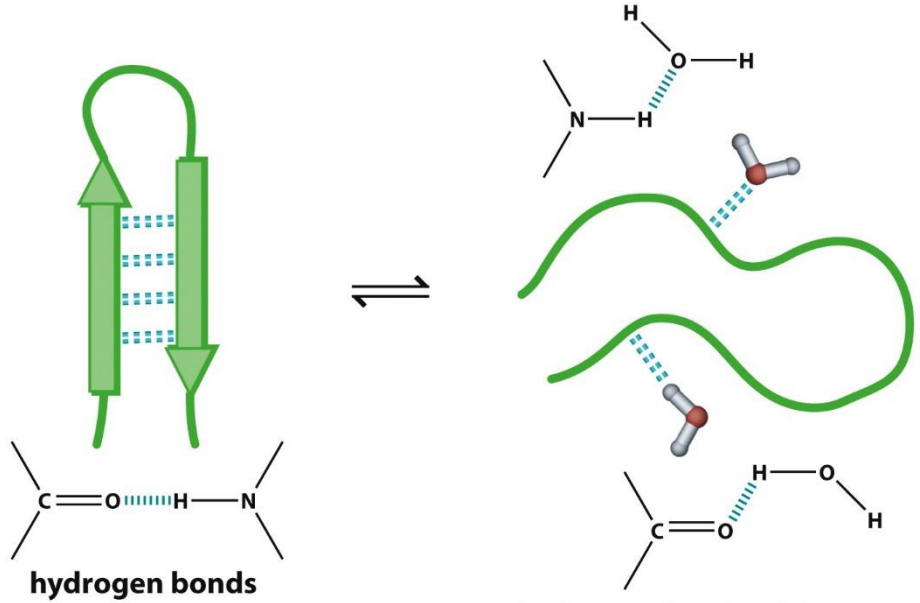


Figure 10.14 The Molecules of Life (© Garland Science 2013)

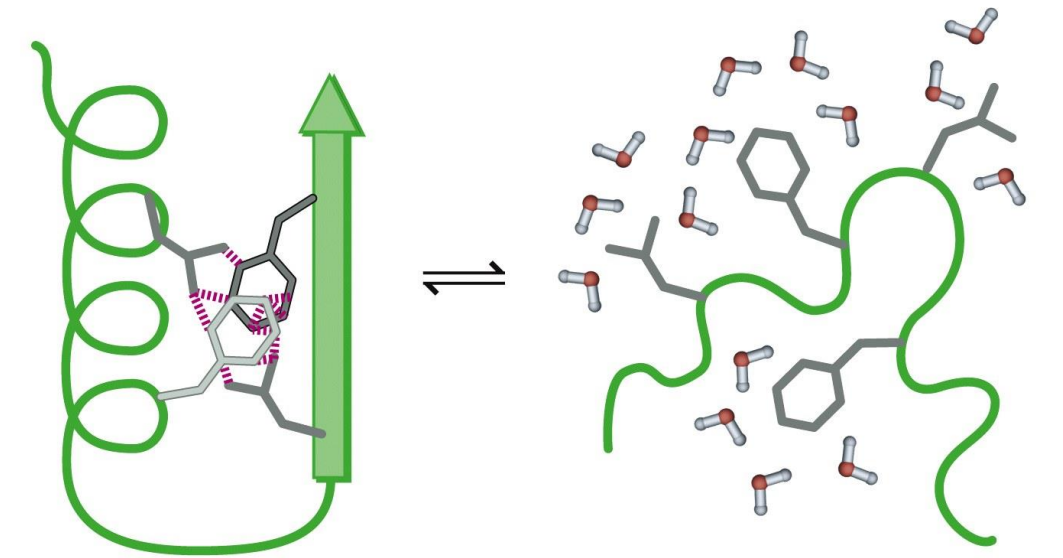


Figure 10.15 The Molecules of Life (© Garland Science 2013)

Entropy change in protein folding

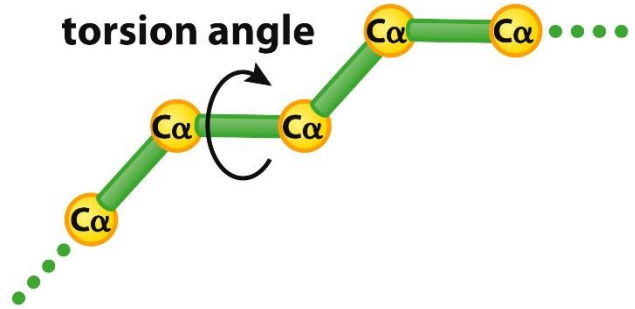


Figure 10.18 The Molecules of Life (© Garland Science 2013)

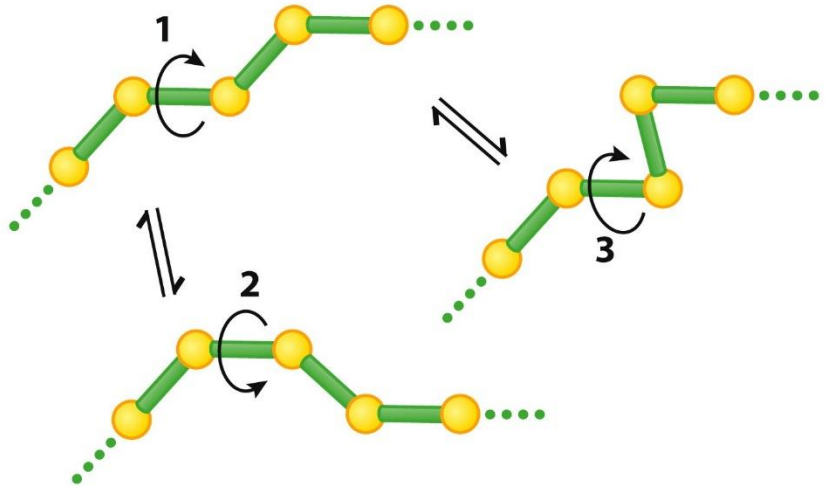


Figure 10.19 The Molecules of Life (© Garland Science 2013)

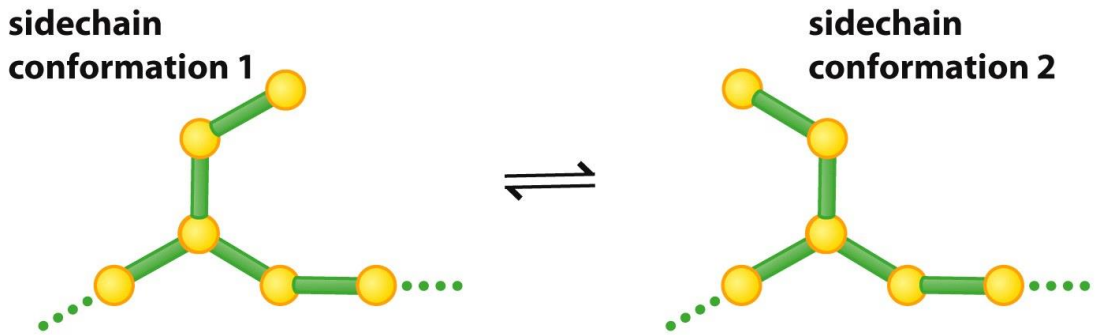


Figure 10.20 The Molecules of Life (© Garland Science 2013)

Entropy contribution from water

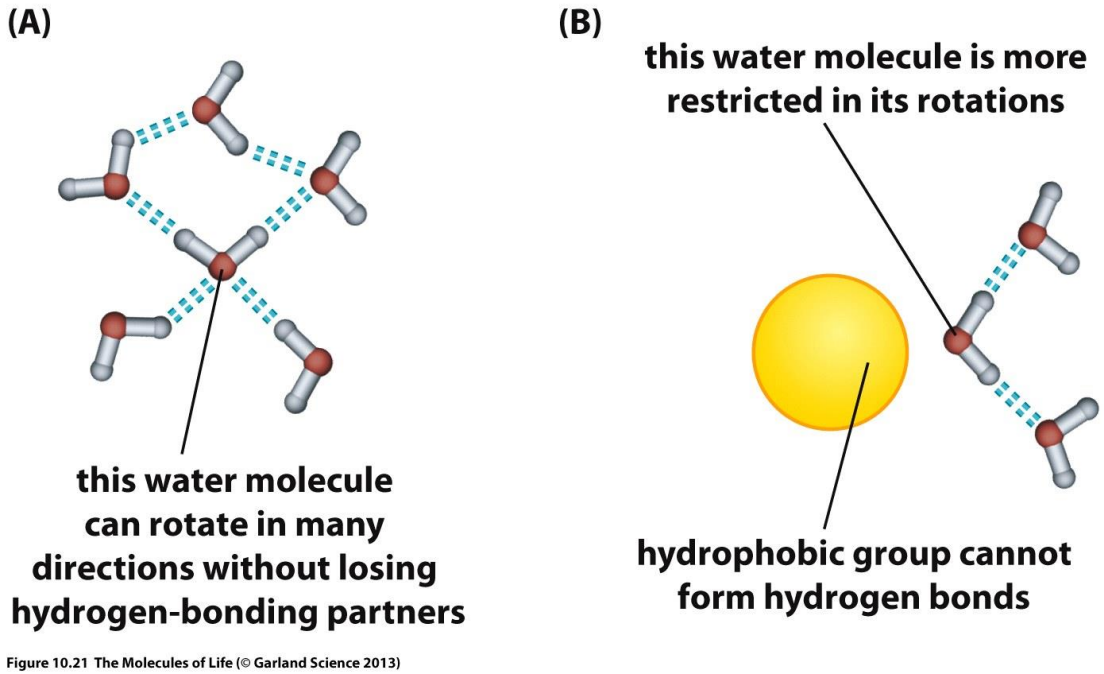


Figure 10.21 The Molecules of Life (© Garland Science 2013)

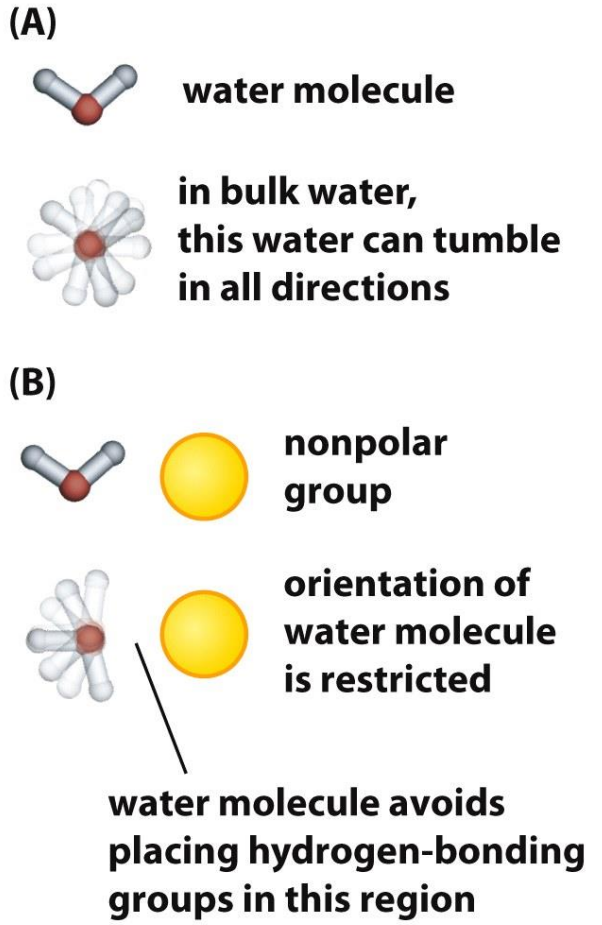


Figure 10.22 The Molecules of Life (© Garland Science 2013)

Protein folding

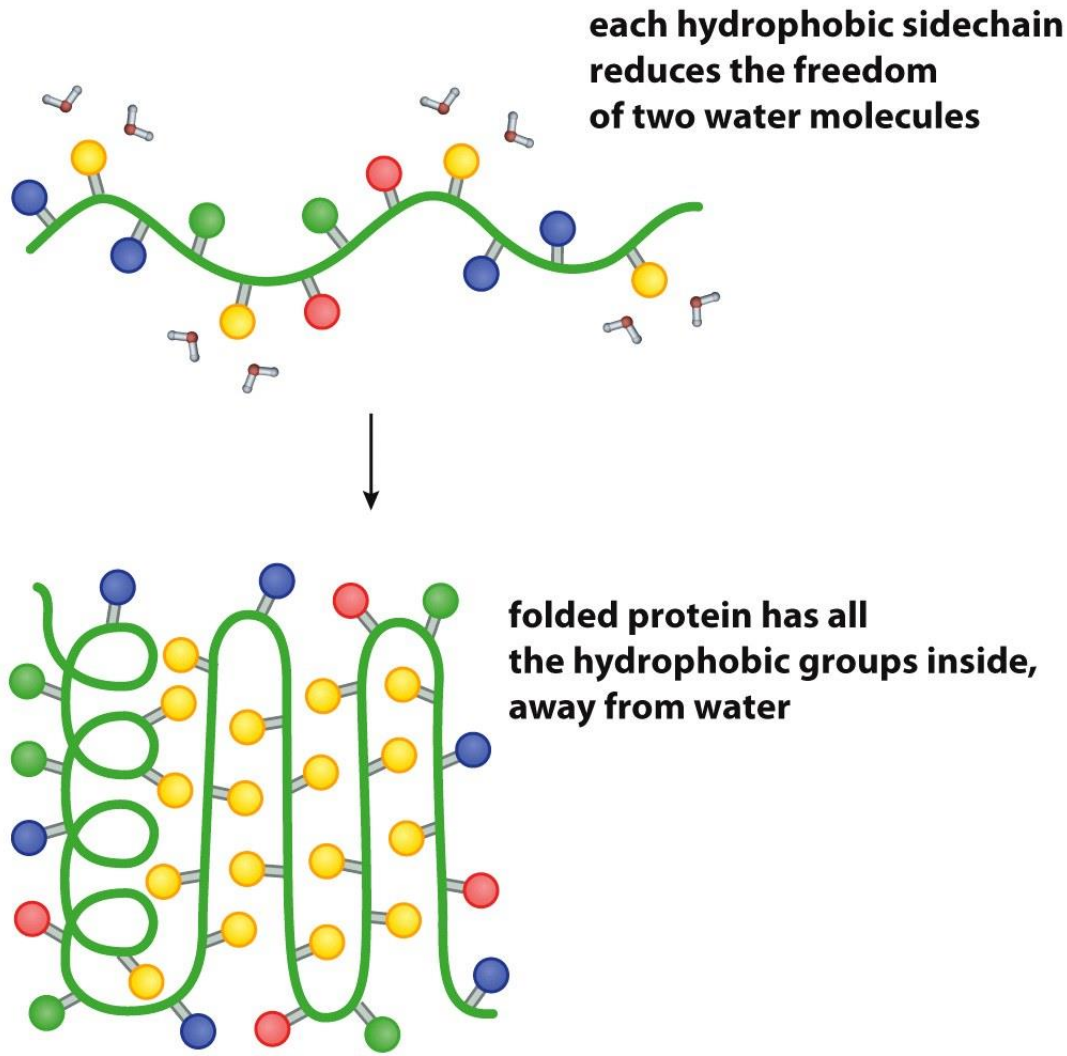
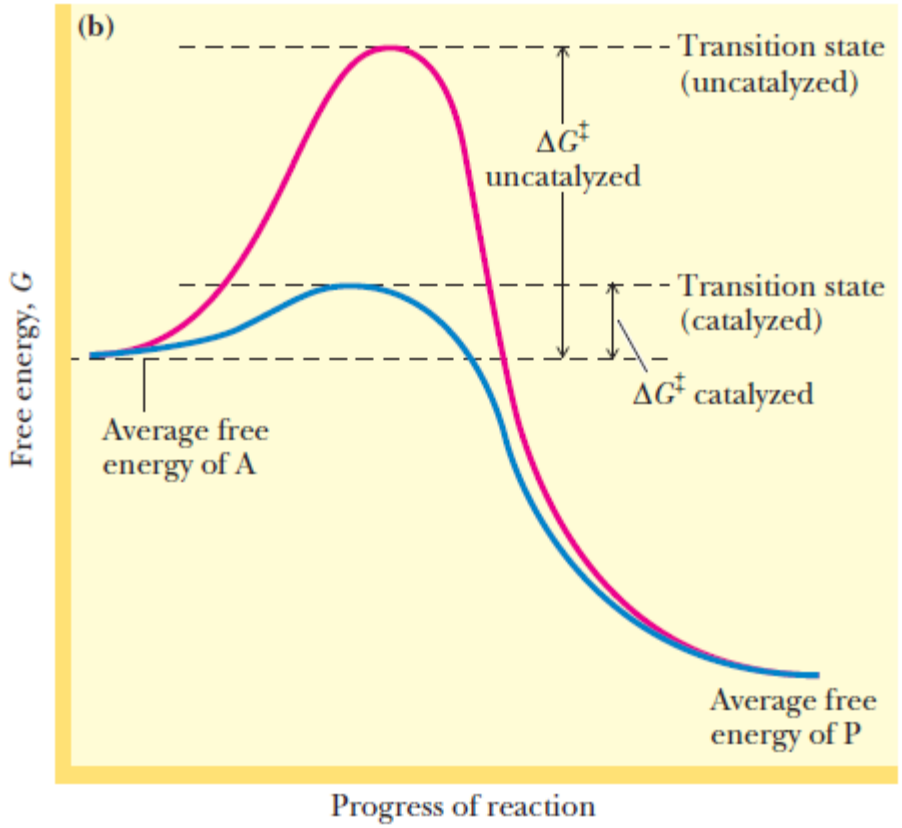


Figure 10.23 The Molecules of Life (© Garland Science 2013)

Enzyme mechanism

Mechanism :Catalysts Lower the Free Energy of Activation for a Reaction



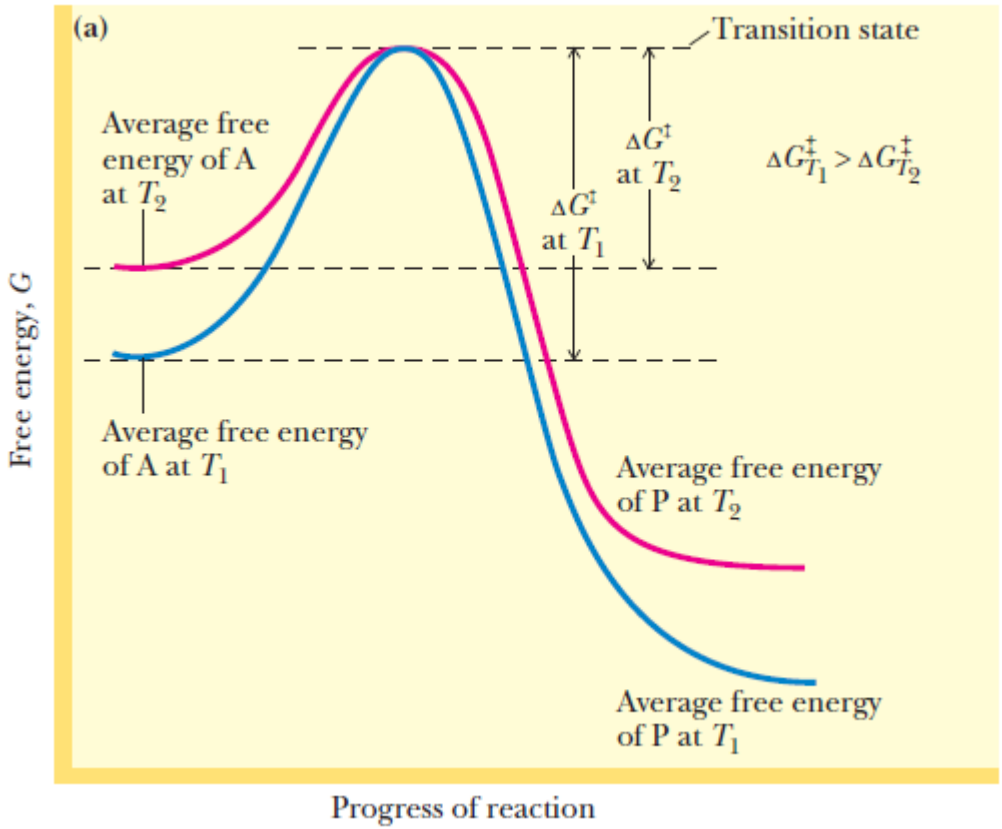


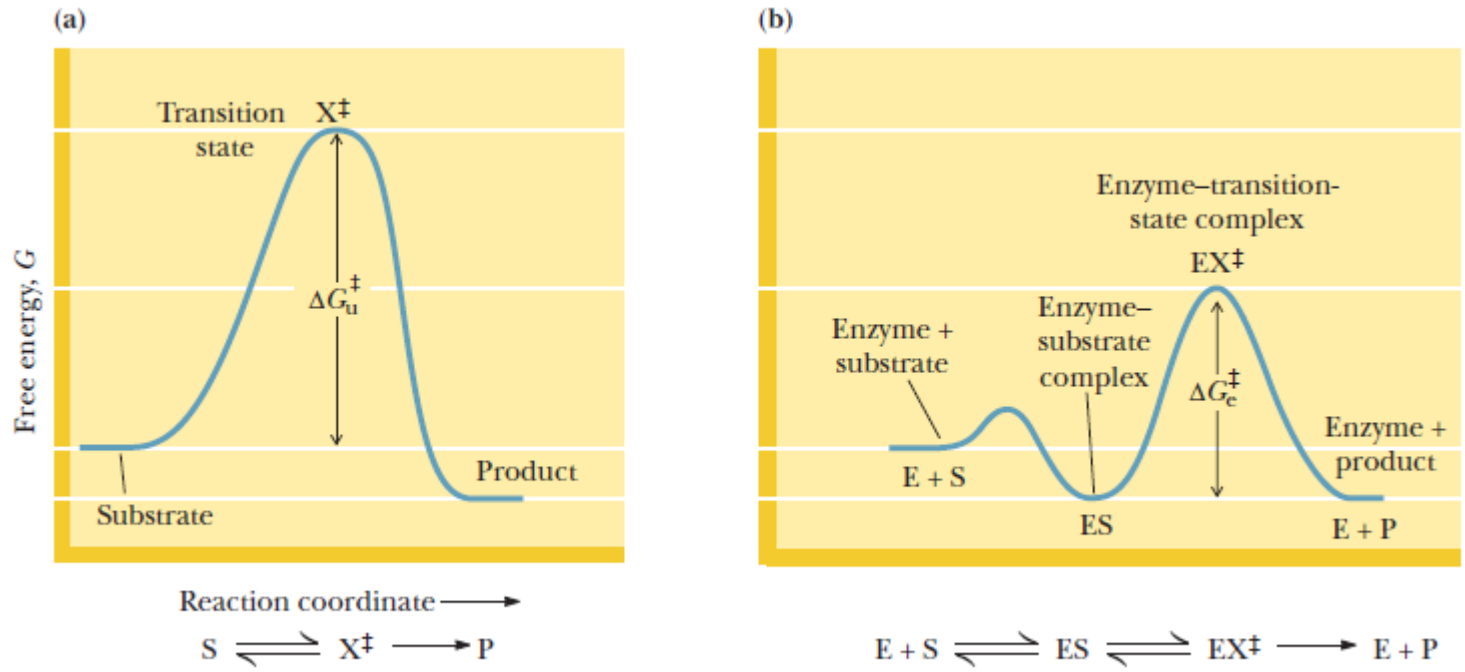
TABLE 14.1 A Comparison of Enzyme-Catalyzed Reactions and Their Uncatalyzed Counterparts

Reaction	Enzyme	Uncatalyzed Rate, v_u (sec^{-1})	Catalyzed Rate, v_e (sec^{-1})	v_e/v_u
Fructose-1,6-bisP $\longrightarrow$ fructose-6-P + P_i	Fructose-1,6-bisphosphatase	2×10^{-20}	21	1.05×10^{21}
$(Glucose)_n + H_2O \longrightarrow (glucose)_{n-2}$ + maltose	β-amylase	1.9×10^{-15}	1.4×10^{3}	7.2×10^{17}
DNA, RNA cleavage	Staphylococcal nuclease	7×10^{-16}	95	1.4×10^{17}
$CH_3—O—PO_3^{2-} + H_2O \longrightarrow CH_3OH + HPO_4^{2-}$	Alkaline phosphatase	1×10^{-15}	14	1.4×10^{16}
$H_2N—\overset{\overset{O}{\|}}{C}—NH_2 + 2\,H_2O + H^+ \longrightarrow 2\,NH_4^+ + HCO_3^-$	Urease	3×10^{-10}	3×10^{4}	1×10^{14}
$R—\overset{\overset{O}{\|}}{C}—O—CH_2CH_3 + H_2O \longrightarrow RCOOH + HOCH_2CH_3$	Chymotrypsin	1×10^{-10}	1×10^{2}	1×10^{12}
Glucose + ATP $\longrightarrow$ Glucose-6-P + ADP	Hexokinase	$<1 \times 10^{-13}$	1.3×10^{-3}	$>1.3 \times 10^{10}$
$CH_3CH_2OH + NAD^+ \longrightarrow CH_3\overset{\overset{O}{\|}}{C}H + NADH + H^+$	Alcohol dehydrogenase	$<6 \times 10^{-12}$	2.7×10^{-5}	$>4.5 \times 10^{6}$
$CO_2 + H_2O \longrightarrow HCO_3^- + H^+$	Carbonic anhydrase	10^{-2}	10^{5}	1×10^{7}
Creatine + ATP $\longrightarrow$ Cr-P + ADP	Creatine kinase	$<3 \times 10^{-9}$	4×10^{-5}	$>1.33 \times 10^{4}$

Adapted from Koshland, D., 1956. Molecular geometry in enzyme action. *Journal of Cellular Comparative Physiology,* Supp. 1, **47**:217; and Wolfenden, R., 2006. Degrees of difficulty of water-consuming reactions in the absence of enzymes. *Chemical Reviews* **106**:3379–3396.

In all chemical reactions, the reacting atoms or molecules pass through a state that is intermediate in structure between the reactant(s) and the product(s). Consider the transfer of a proton from a water molecule to a chloride anion:

$$\underset{\text{Reactants}}{H{-}O{-}H + Cl^-} \rightleftharpoons \underset{\text{Transition state}}{H{-}O^{\delta-}\cdots H\cdots Cl^{\delta-}} \rightleftharpoons \underset{\text{Products}}{HO^- + H{-}Cl}$$



Without enzyme

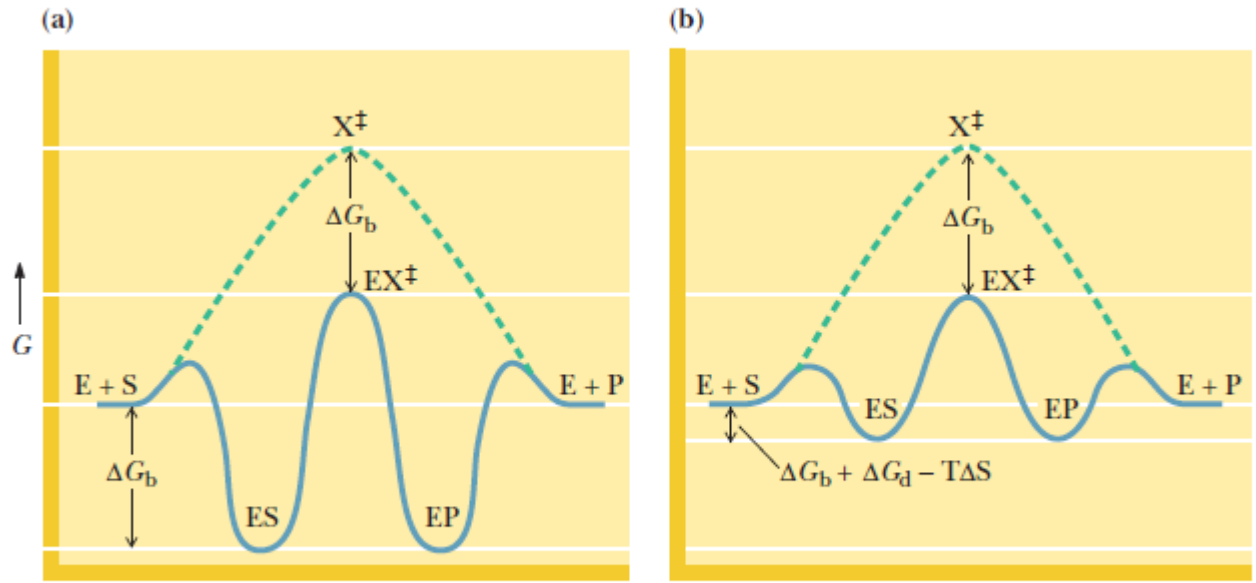
With enzyme

$$E + S \rightleftharpoons ES \rightleftharpoons EX^{\ddagger} \longrightarrow E + P$$

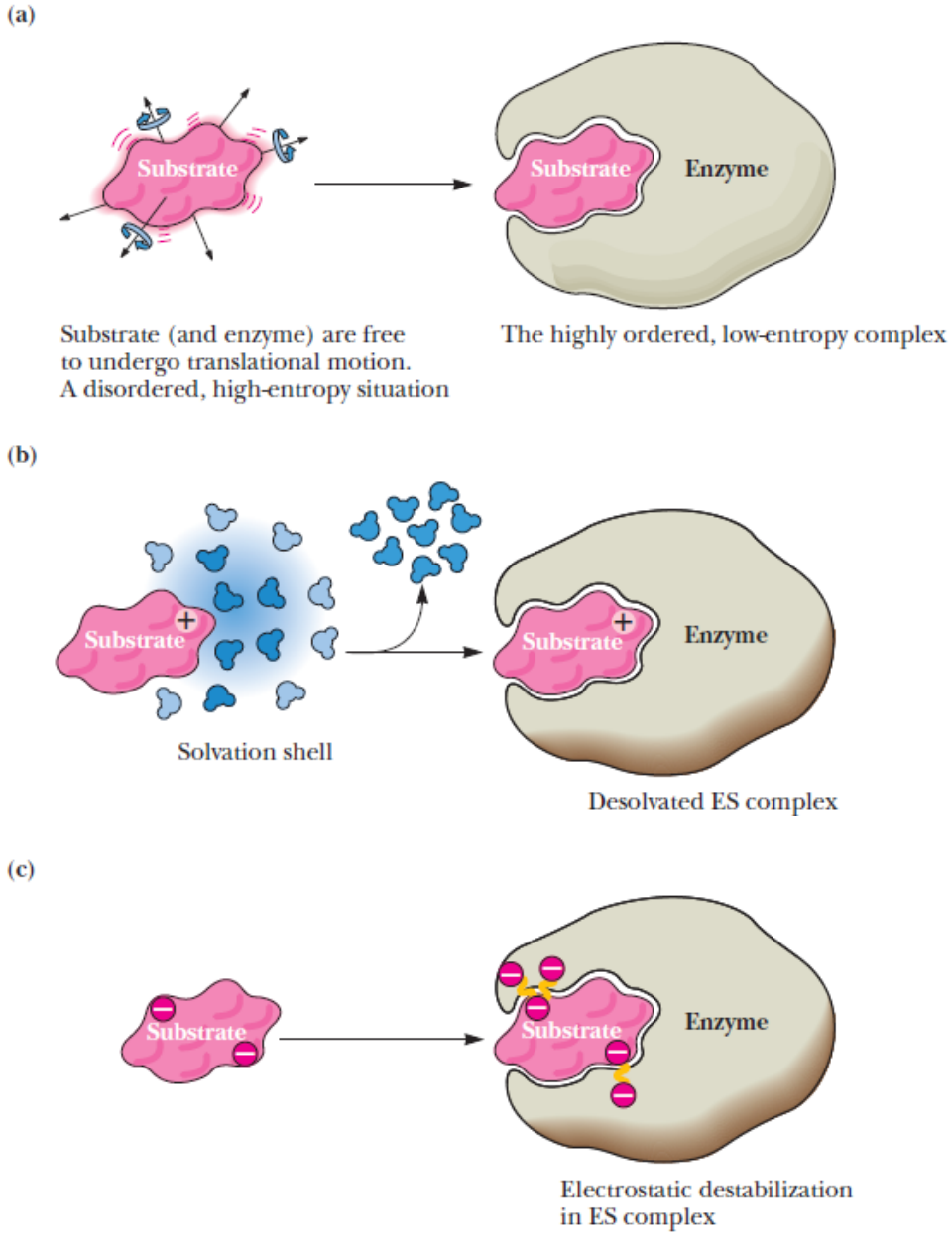
$$K_S = \frac{[E][S]}{[ES]}$$

$$K_T = \frac{[E][X^{\ddagger}]}{[EX^{\ddagger}]}$$

$$k_e/k_u \approx K_S/K_T$$



Partially de-stabilize
ES complex

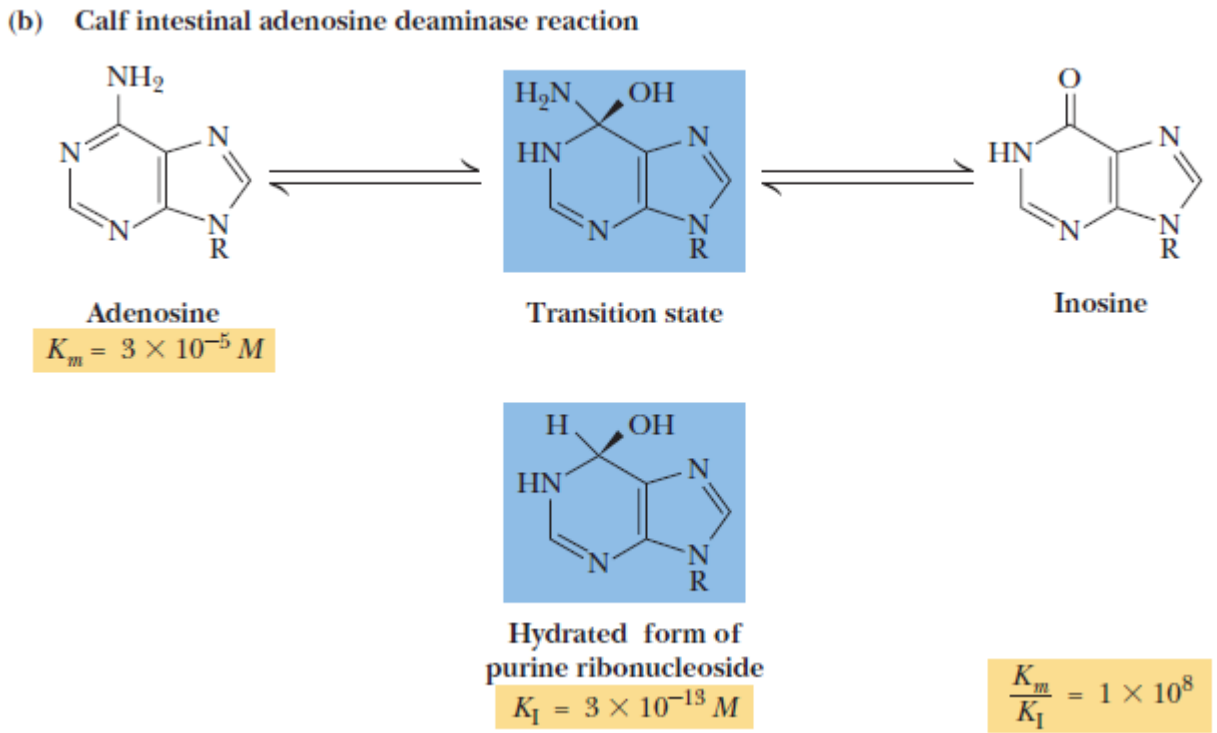


How analogs of transition state exploited?

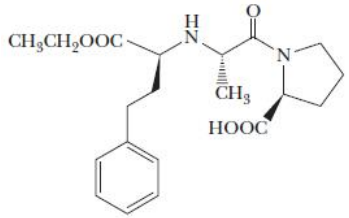
The value of K_T for fructose-1,6-bisphosphatase is an astounding 7 x 10^{-26} M!

This low value for binding constant means very tight binding between enzyme and transition complex

If you can mimic the transition state with similar compound, it will bind the enzyme complex very tightly. This idea is exploited very well and many know drug today have designed on this principle

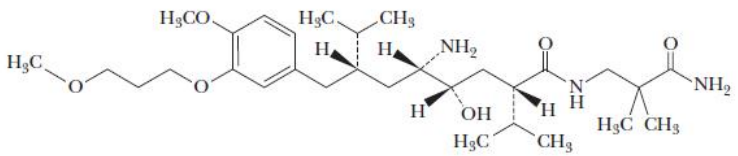


Angiotensin Concerting Enzyme (ACE)



Enalapril

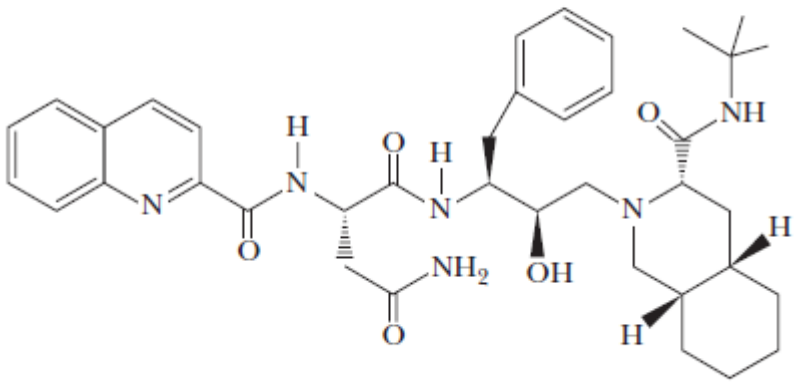
Courtesy of James Gathany/CDC



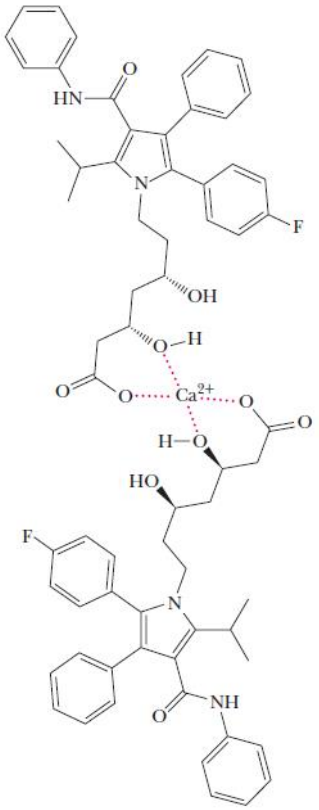
Aliskiren

Protease Inhibitors Are AIDS Drugs

Crixivan (indinavir) by Merck, Invirase (saquinavir) by Roche, and similar "protease inhibitor" drugs are transition-state analogs for the HIV-1 protease, discussed on pages 470–471.



Saquinavir



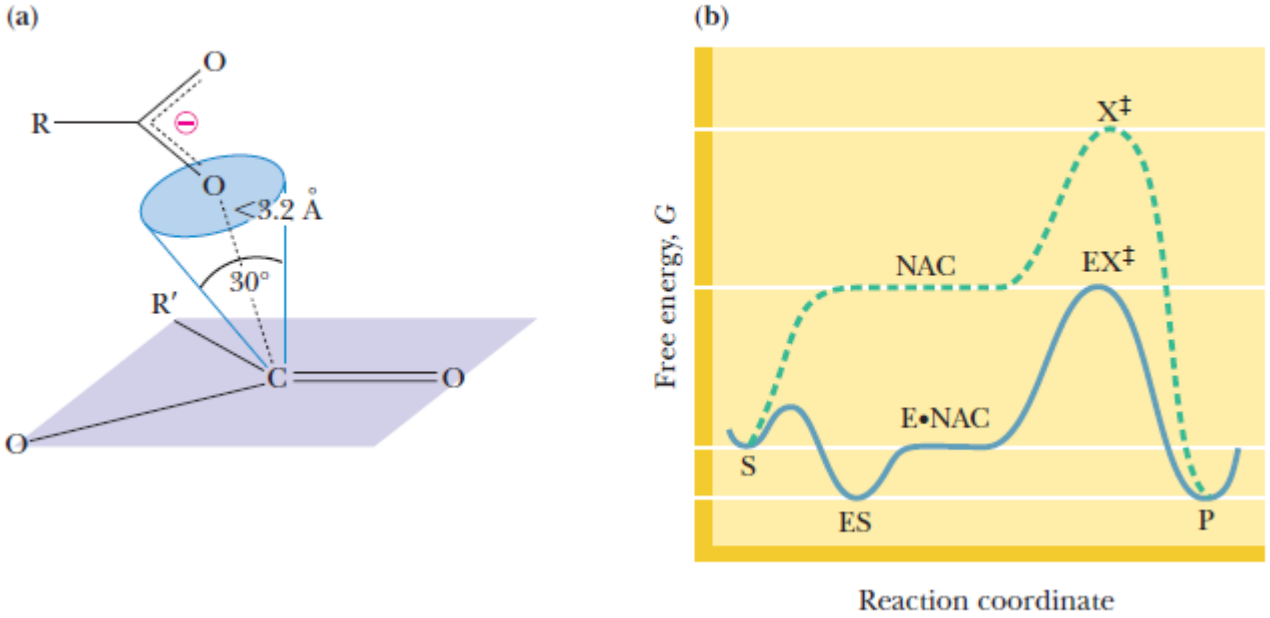
Atorvastatin (Lipitor)

© AP Photo/Amy Sancetta, File

HMG-CoA reductase

Near attack conformations

Reacting atoms are in *Van der waals* contact and at an angle resembling the bond to be formed



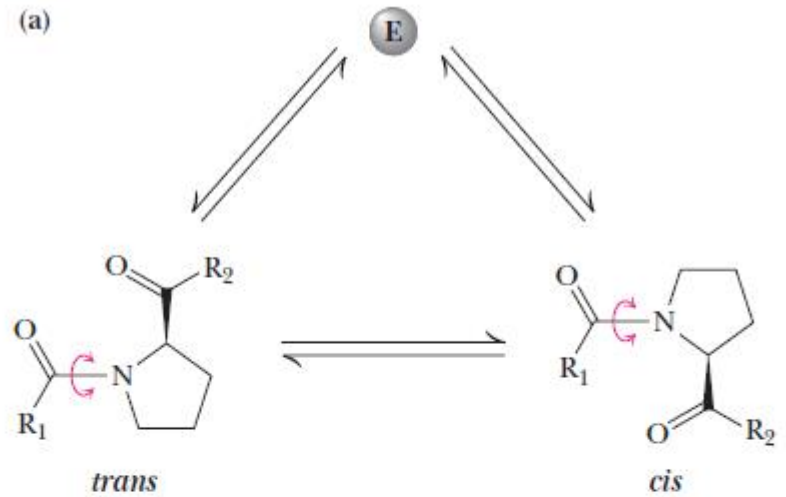
For reactions involving bonding between O, N, C & S atoms: NAC are characterized as Distance within 3.2 Å and ± 15 angle

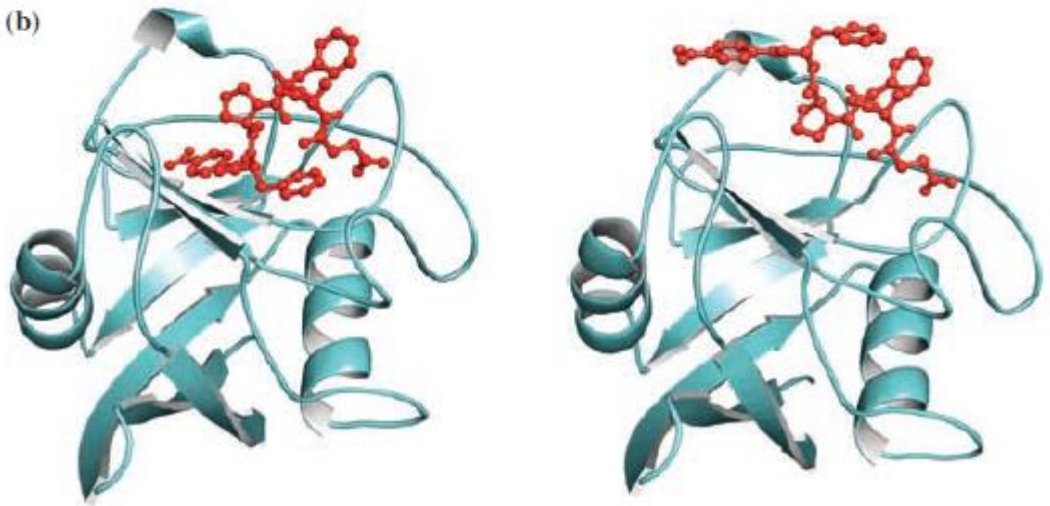
What exactly happens at protein active site??

Different amino-acids in protein active site are in constant motions and they assist in

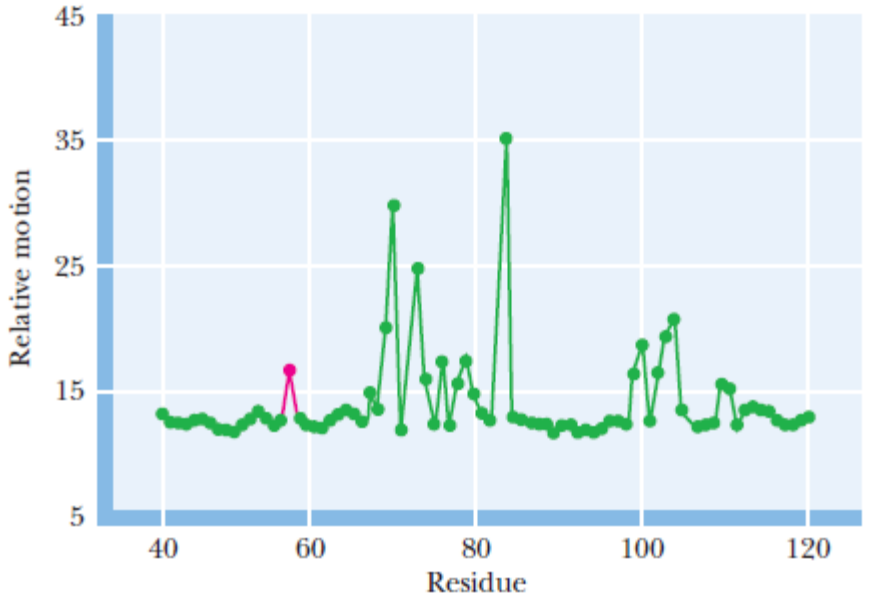
1. Substrate binding
2. Bring catalytic group into position around a substrate
3. Induce formation of NAC
4. Assist in bond making and bond breaking
5. Facilitate conversion of substrate to product

example





Human cyclophilin A is a prolyl isomerase, which catalyzes the interconversion between *trans* and *cis* conformations of proline in peptides.



This network extends from the active site to the surface of the protein, and the motions in this network span time scales of femtoseconds to milliseconds. Such extensive networks of motion make it likely that the entire folded structure of the protein may be involved in catalysis at the active site.

Covalent Catalysis

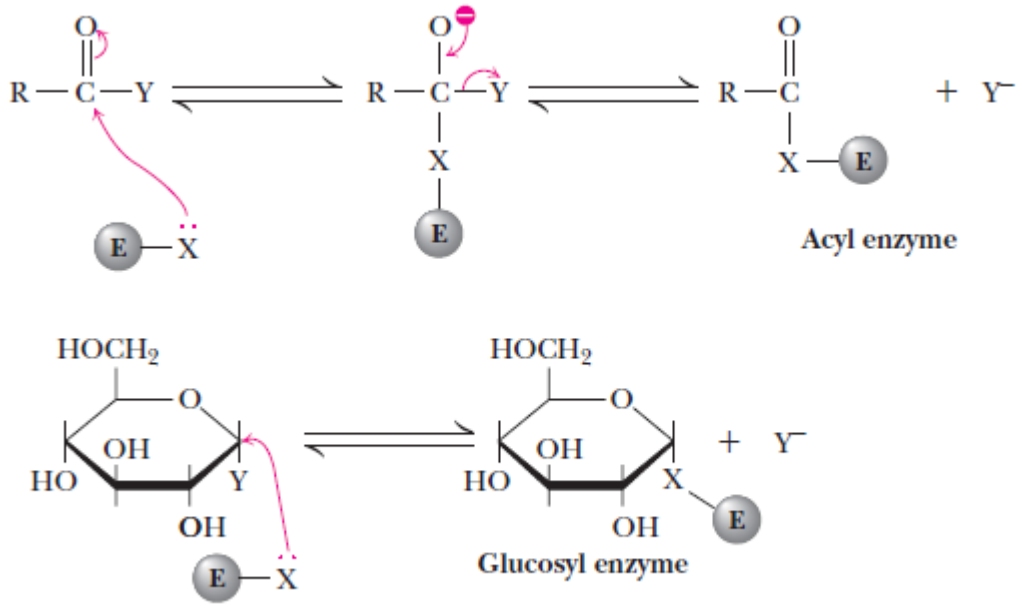
Some enzyme reactions derive much of their rate acceleration from the formation of **covalent bonds** between enzyme and substrate. Consider the reaction:

$$BX + Y \longrightarrow BY + X$$

and an enzymatic version of this reaction involving formation of a **covalent intermediate:**

$$BX + Enz \longrightarrow E{:}B + X + Y \longrightarrow Enz + BY$$

examples



Digestive serine protease

Ex., trypsin, chymotrypsin, thrombin, tissue plasminogen activator

Catalytic mechanism based on active site serine

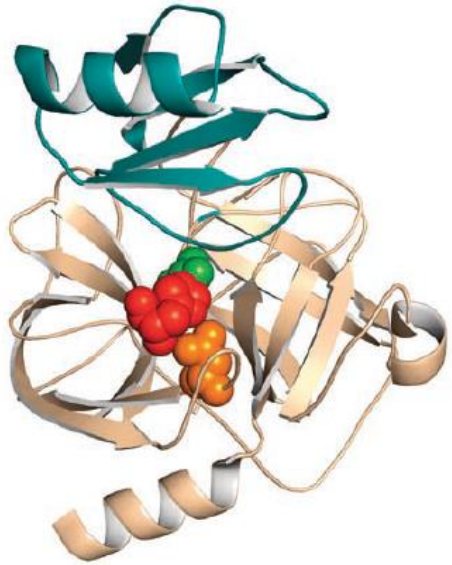


FIGURE 14.16 Structure of chymotrypsin (white) in a complex with eglin C (blue ribbon structure), a target protein. The residues of the catalytic triad (His^{57}, Asp^{102}, and Ser^{195}) are highlighted. His^{57} (red) is flanked by Asp^{102} (gold) and by Ser^{195} (green). The catalytic site is filled by a peptide segment of eglin. Note how close Ser^{195} is to the peptide that would be cleaved in the chymotrypsin reaction (pdb id = 1ACB).

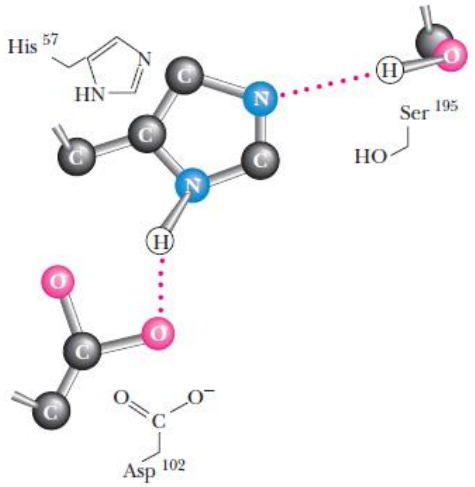
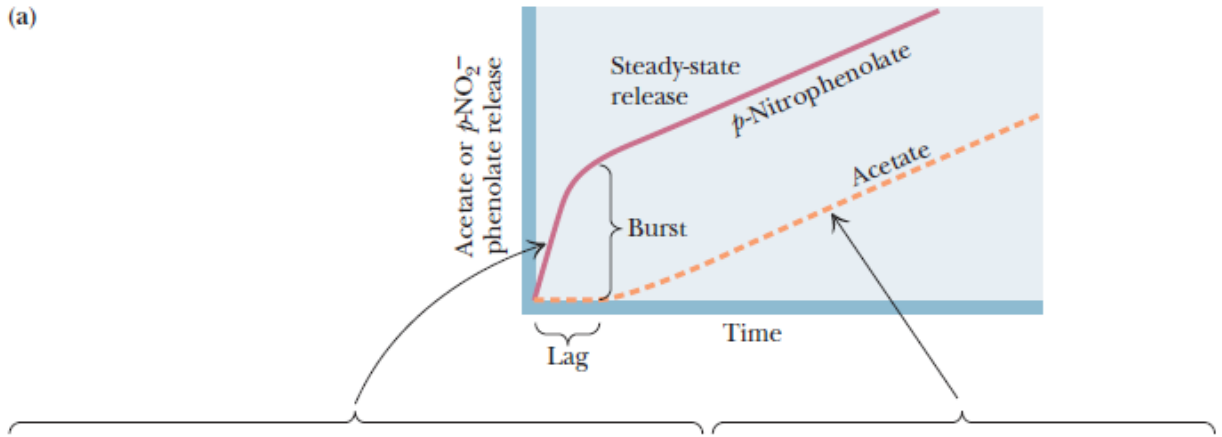
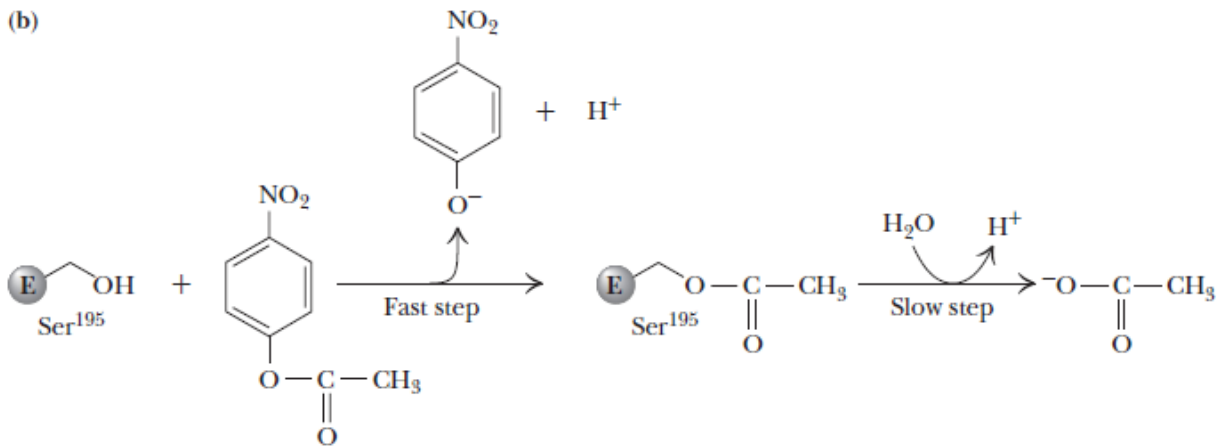
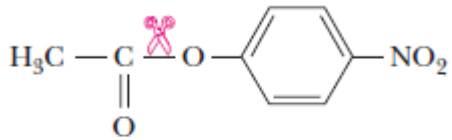


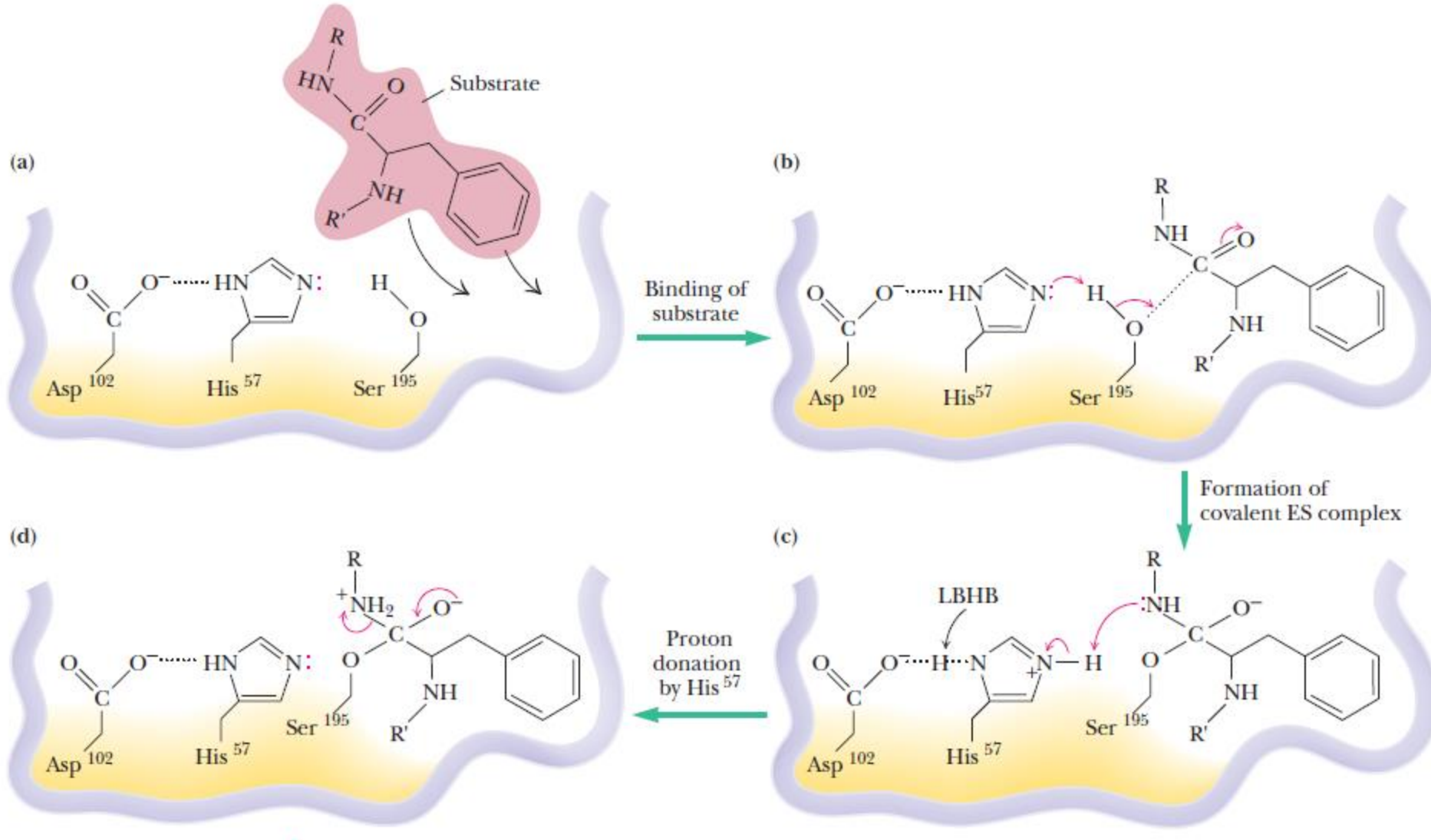
FIGURE 14.17 The catalytic triad of chymotrypsin.

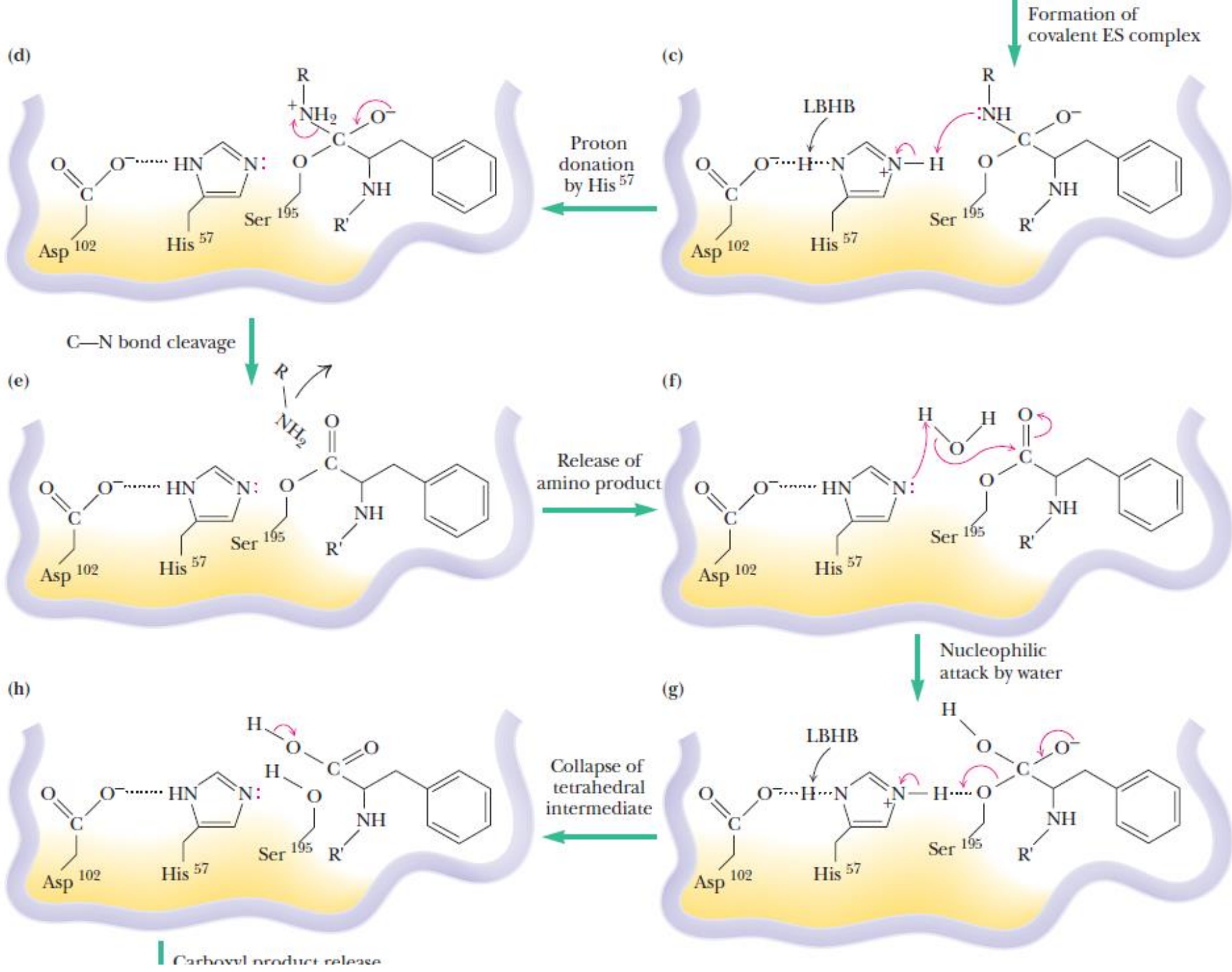






p-Nitrophenylacetate


(a)
Substrate
Asp 102
His 57
Ser 195
Binding of substrate
(b)
Asp 102
His57
Ser 195
Formation of covalent ES complex
(c)
LBHB
Asp 102
His 57
Ser 195
Proton donation by His 57
(d)
Asp 102
His 57
Ser 195


(d)
(c)
Formation of covalent ES complex
Proton donation by His 57
LBHB
C—N bond cleavage
(e)
(f)
Release of amino product
Nucleophilic attack by water
(h)
(g)
Collapse of tetrahedral intermediate
Asp 102
His 57
Ser 195
R'
NH
NH2

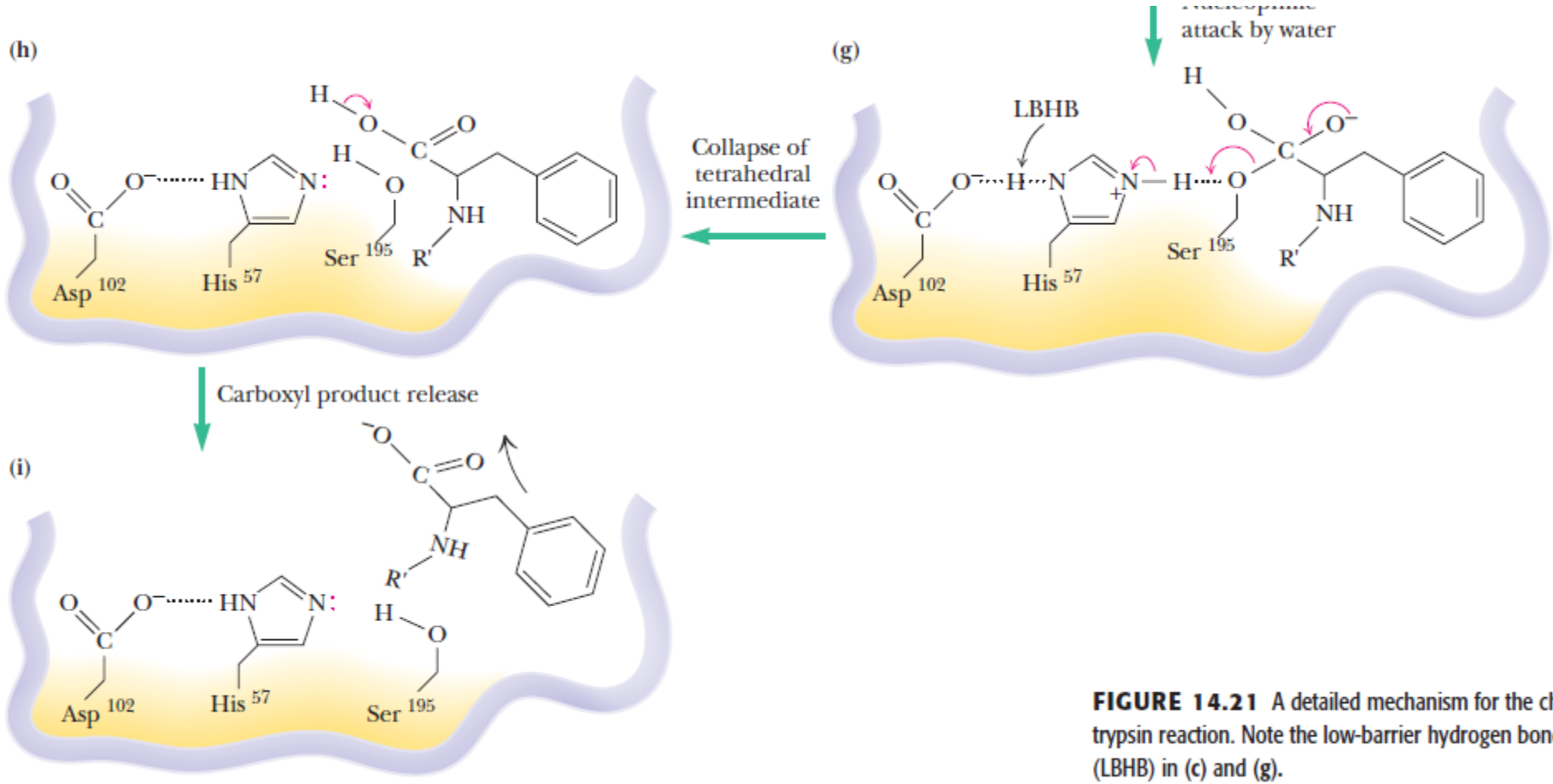
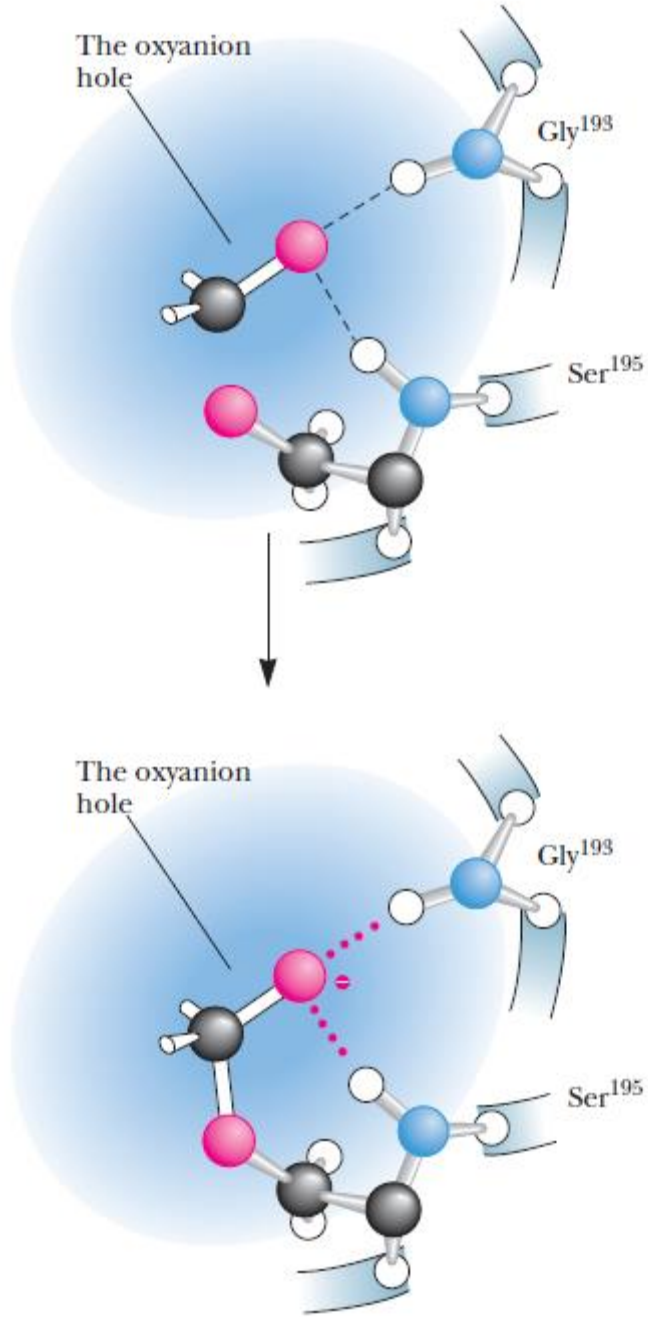


FIGURE 14.21 A detailed mechanism for the chymotrypsin reaction. Note the low-barrier hydrogen bond (LBHB) in (c) and (g).


The oxyanion hole
Gly193
Ser195
The oxyanion hole
Gly193
Ser195