

# Techniques in Molecular Genetics

**Spectroscopy and  
Enzyme Assays**

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H.E. Schellhorn

# Spectroscopy

- Chromophore
- Molar Extinction Coefficient
- Absorbance
- Transmittance

# Spectroscopy

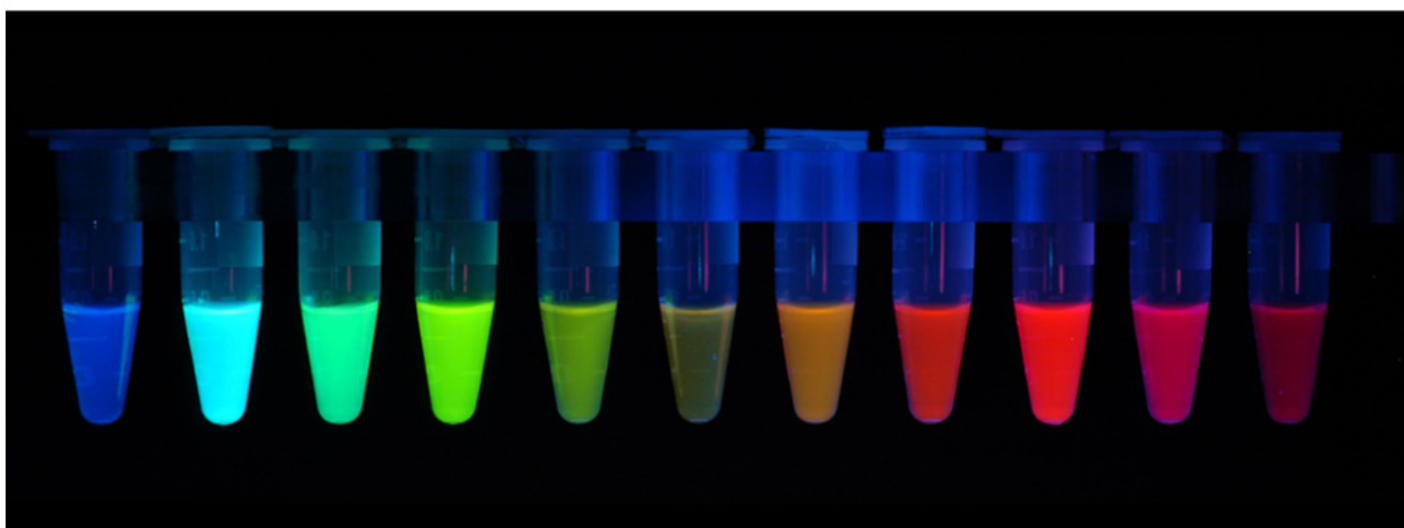
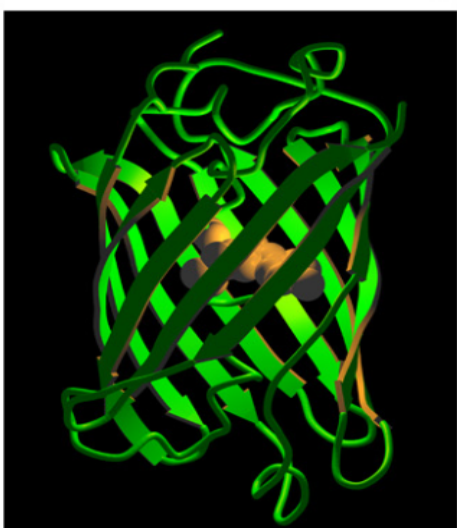
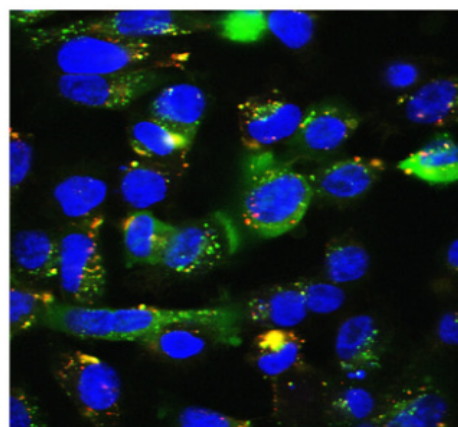
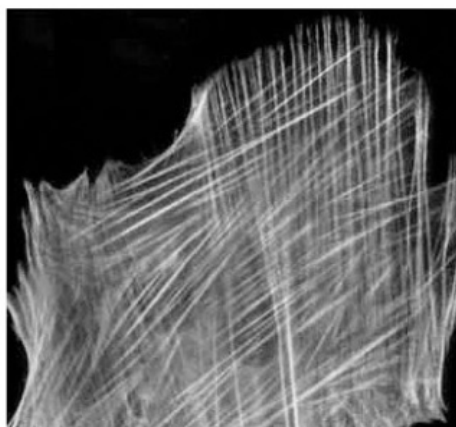
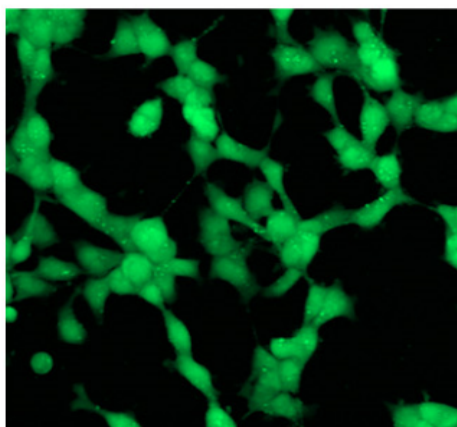
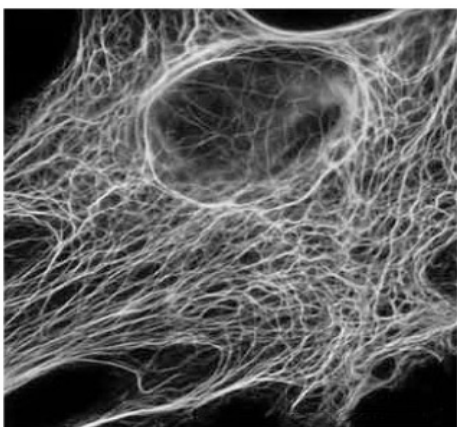
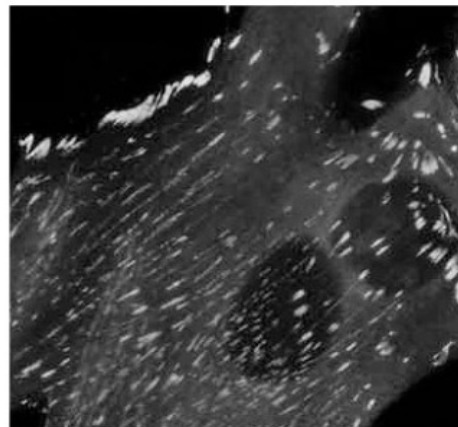
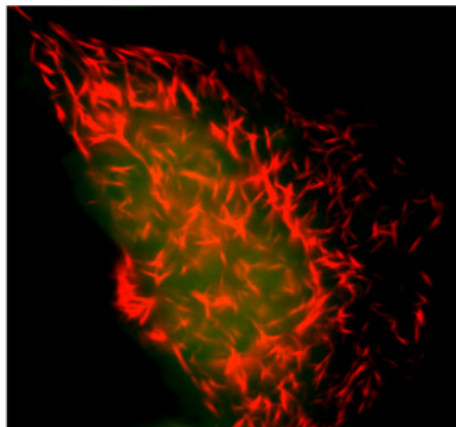
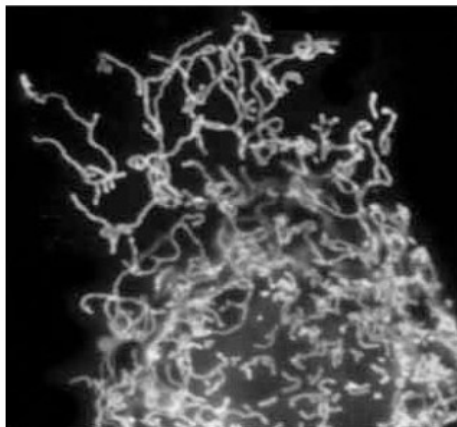
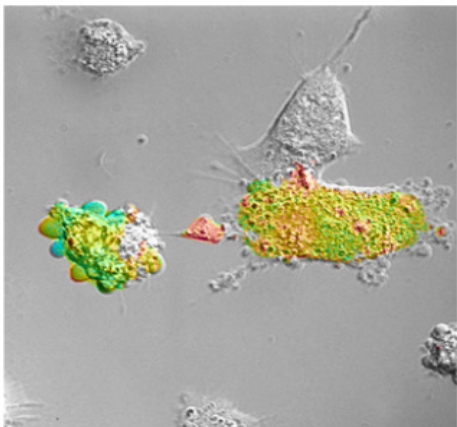
Many biological materials have optical properties that we can exploit for detection and quantitation (e.g. DNA and Protein absorb maximally at 260 and 280 nm respectively...).

# Spectroscopy

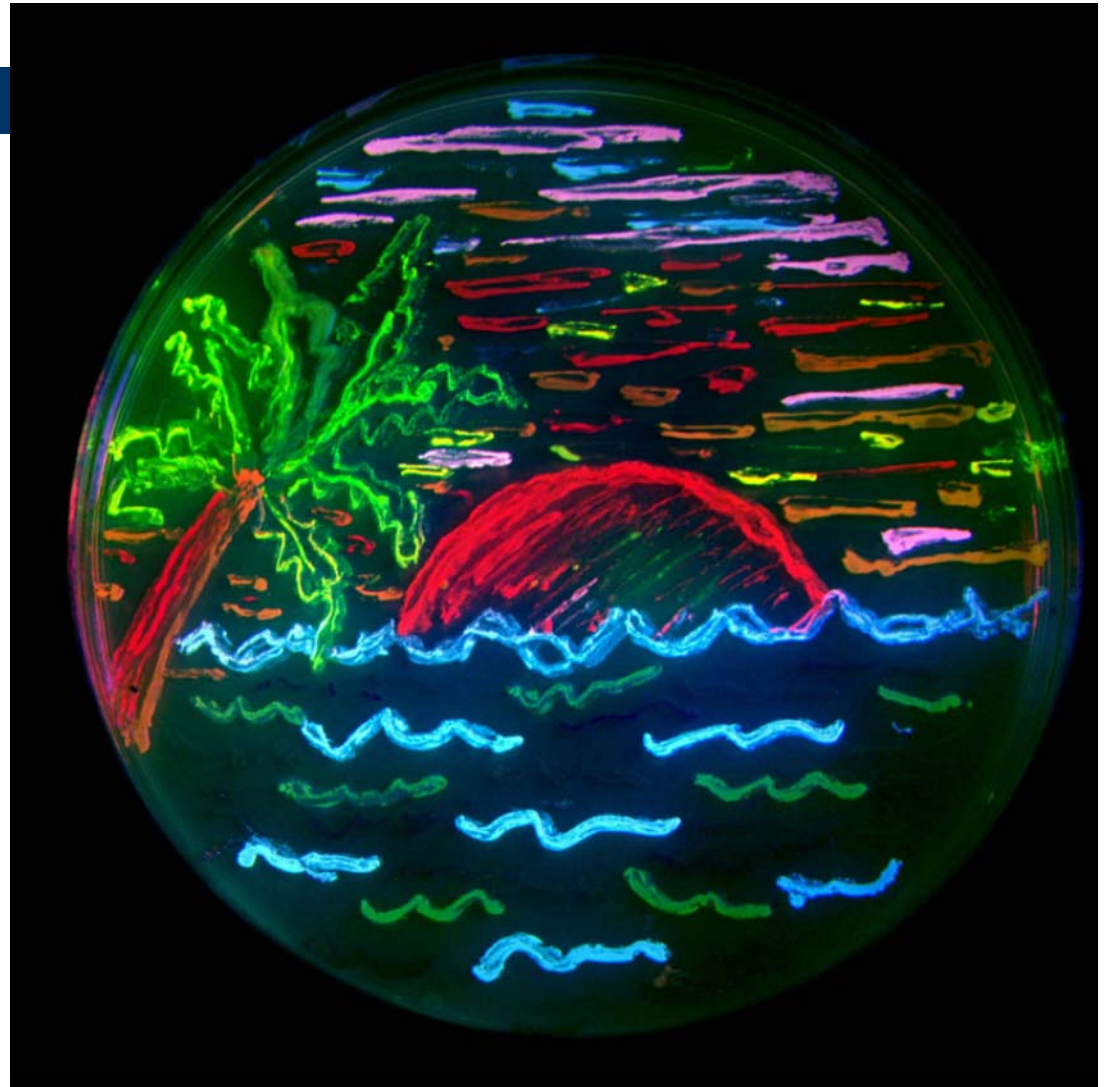
Lots of new developments in the field..

i.e. Roger Y. Tsien won the 2008 Nobel Prize in chemistry "for his discovery and development of the green fluorescent protein (GFP)

.



Also can be used for practicing streaking technique...



# Abbe Limit broken! (after 120 years)

$$\text{Abbe limit } (d) = \lambda / 2 \times \text{NA}$$



Ernst Abbe  
(1840-1905)

## News and Views

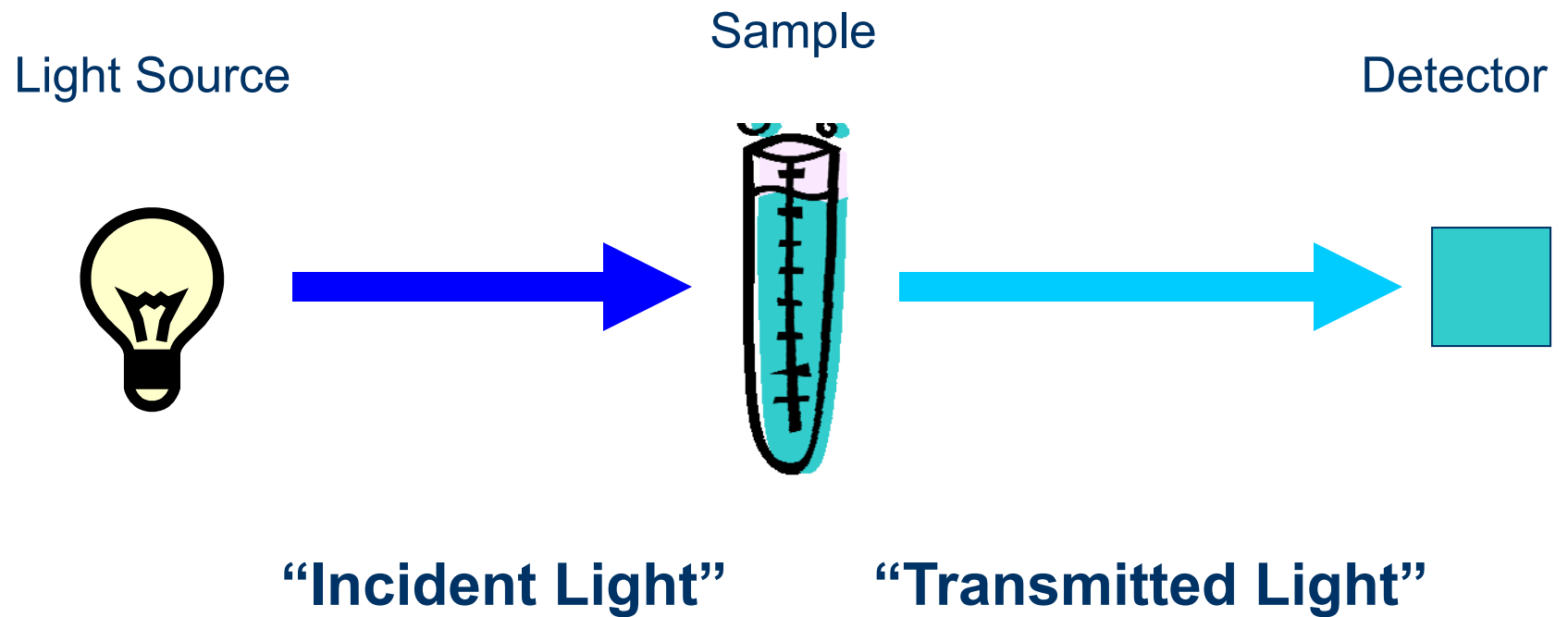
*Nature* **417**, 806-807 (20 June 2002) | [doi:10.1038/417806a](https://doi.org/10.1038/417806a)

Light microscopy: Beyond the diffraction limit?

Ernst H. K. Stelzer

**The wave nature of light manifests itself in diffraction, which hampers attempts to determine the location of molecules. Clever use of microscopic techniques might now be circumventing the 'diffraction limit'.**

# Measuring Absorbance..





# Absorbance

The ratio of light transmitted ( $I$ ) by a sample to the incident light ( $I_0$ ) on the sample is called the *transmittance*  $T$ :

$$T = I/I_0$$

Absorbance ( $A$ ) can be defined as the logarithm (base 10) of the reciprocal of the transmittance:

$$A = -\log T = \log (1/T)$$

# Absorbance and Concentration

In a spectrophotometer, light enters a sample and some fraction of light is absorbed..

## Beer-Lambert law

At a given wavelength, the transmittance (**T**) and absorbance (**A**) depends on the molar concentration (**c**), light path length in centimeters (**L**), and molar absorptivity ( $\epsilon$ ) for the dissolved substance

$$T = 10^{-\epsilon c L} \text{ or } A_{\lambda} = \epsilon c L,$$

$\epsilon$  – *Molar Extinction Coefficient*

$A_{\lambda}$  – Absorbance at a given wavelength ( $\lambda$ )

## Molar Extinction Coefficient (examples)

Transmittance and absorbance are dimensionless, and therefore the units for molar absorptivity cancel with units of measure in concentration and light path. Molar absorptivities have units of  $M^{-1}cm^{-1}$ . Most spectrophotometers use a one cm pathlength so this is invariant....

(important exception-microtiterplate readers)

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# Enzyme Assays and Kinetics

Enzyme	Non-Enzymatic Rate	Enzymatic Rate	Relative Rate
	$k_{\text{non}}$ , (s <sup>-1</sup> )	$k_{\text{cat}}$ (s <sup>-1</sup> )	$k_{\text{cat}} / k_{\text{non}}$
Cyclophilin	$2.8 \times 10^{-2}$	$1.3 \times 10^4$	$4.6 \times 10^5$
Carbonic anhydrase	$1.3 \times 10^{-1}$	$1.0 \times 10^5$	$7.7 \times 10^6$
Chymotrypsin	$4.0 \times 10^{-9}$	$4.0 \times 10^{-2}$	$1.0 \times 10^7$
Triosephosphate isomerase	$6.0 \times 10^{-7}$	$2.0 \times 10^3$	$3.0 \times 10^9$
Fumarase	$2.0 \times 10^{-8}$	$2.0 \times 10^3$	$1.0 \times 10^{11}$
Adenosine deaminase	$1.8 \times 10^{10}$	370	$2.1 \times 10^{12}$
Urease	$3.0 \times 10^{-10}$	$3.0 \times 10^1$	$1.0 \times 10^{14}$
Alkaline phosphatase	$1.0 \times 10^{-15}$	$1.0 \times 10^2$	$1.0 \times 10^{17}$

Source: various

## Basics of enzyme kinetics: unit definitions

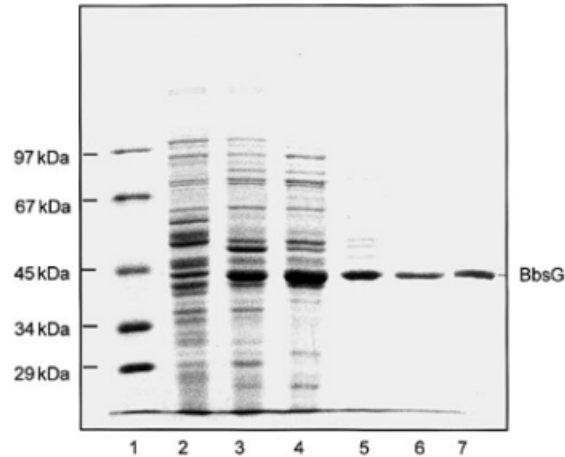
**Rate of reaction** = moles of substrate consumed/product formed  
(mol L<sup>-1</sup> s<sup>-1</sup>)

**Enzyme activity** = moles converted per unit time = rate x volume  
SI unit                      **katal**                      1 katal = 1 mol s<sup>-1</sup>  
   **unit (U)**                      1 unit = μmol min<sup>-1</sup>

**Specific activity** = moles converted per unit time per unit mass of enzyme → enzyme purification  
katal kg<sup>-1</sup> / μmol mg<sup>-1</sup> min<sup>-1</sup> / μmol μg<sup>-1</sup>min<sup>-1</sup>

**Turnover number** = moles converted per unit time  
per mole of enzyme (s<sup>-1</sup>) K<sub>cat</sub>

# Enzyme purification and specific activity

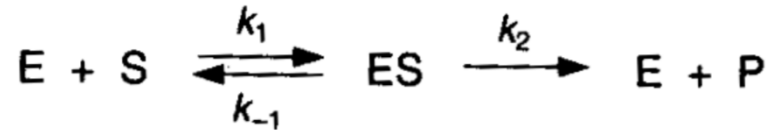


**Fig.2** SDS-PAGE of protein fractions containing (*R*)-benzylsuccinyl-CoA dehydrogenase (*BbsG*) activity. Coomassie-blue-stained SDS-polyacrylamide gel (10% polyacrylamide; w/v). Lanes: 1 Marker, 2 control extract of *Escherichia coli* lacking BCDH, 3 *E. coli* extract containing recombinant BCDH, 4 pooled fractions from Q sepharose chromatography, 5 pooled fractions from hydroxyapatite chromatography, 6 pooled fractions from butyl-TSK chromatography, 7 pooled fractions from gel filtration. The masses of marker proteins are indicated in the left margin

**Table 1** Purification of recombinant (*R*)-benzylsuccinyl-CoA dehydrogenase. One unit corresponds to 1  $\mu\text{mol}$  benzylsuccinyl-CoA oxidised  $\text{min}^{-1}$

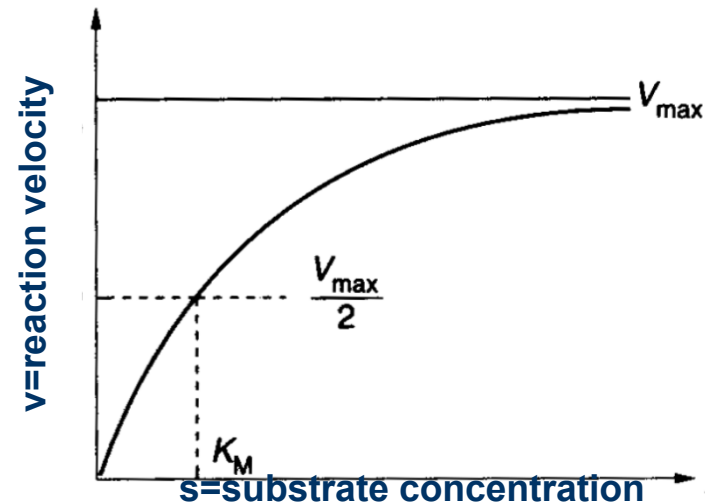
Fraction	Volume (ml)	Protein (mg)	Activity (U)	Specific activity ( $\text{U mg}^{-1}$ )	Enrichment (fold)	Yield (%)
Extract	12.5	563	426	0.76	1	100
Q-Sepharose	45	162	178	1.1	1.4	42
Hydroxyapatite	72	97.2	175	1.8	2.4	41
Butyl-Fractogel	36	16.2	68	4.2	5.5	16
Superdex HR200	36	3.6	23	6.4	8.4	5.3

# Basics of enzyme kinetics: Michaelis-Menten



Michaelis-Menten

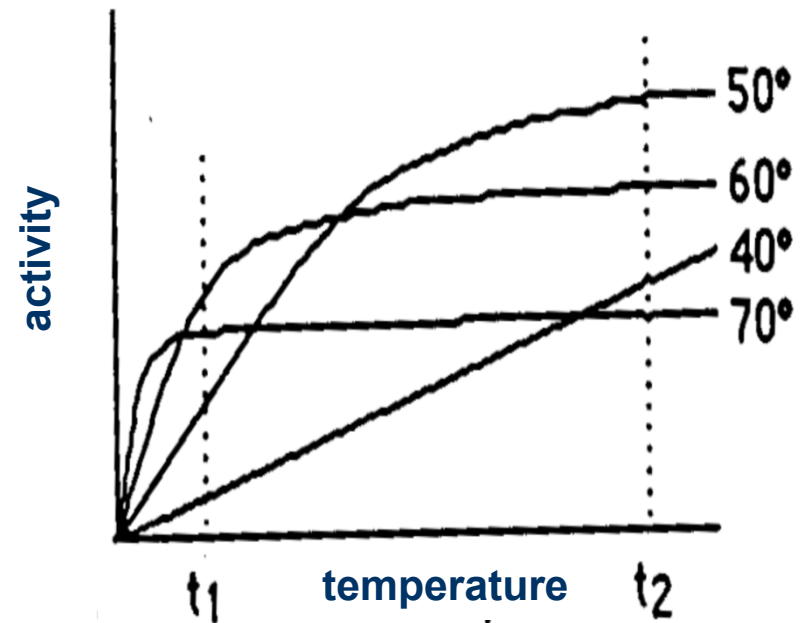
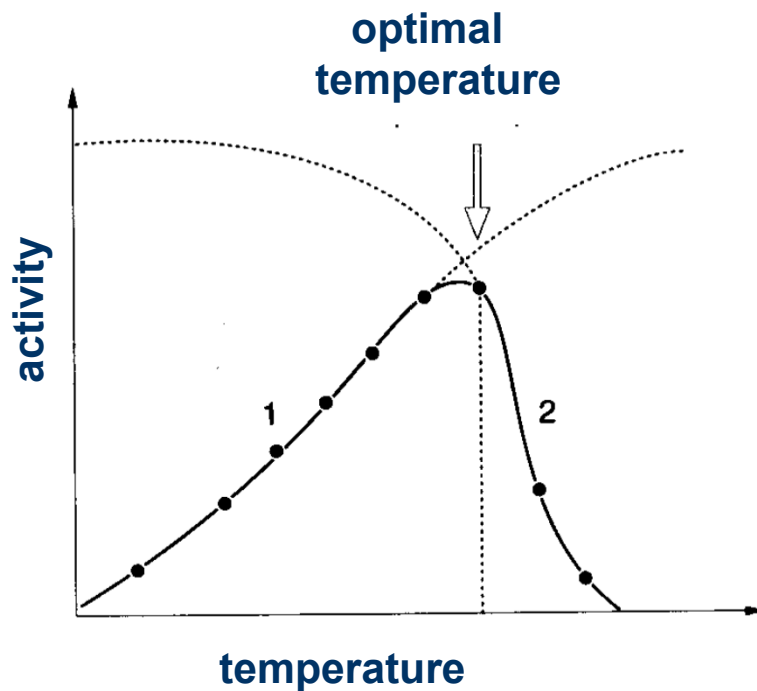
$$V = \frac{V_{\max} \cdot [S]}{K_M + [S]}$$



$$K_M \Rightarrow V_{\max}/2$$



# Factors affecting enzyme activities: Temperature



Which enzyme is most thermostable?

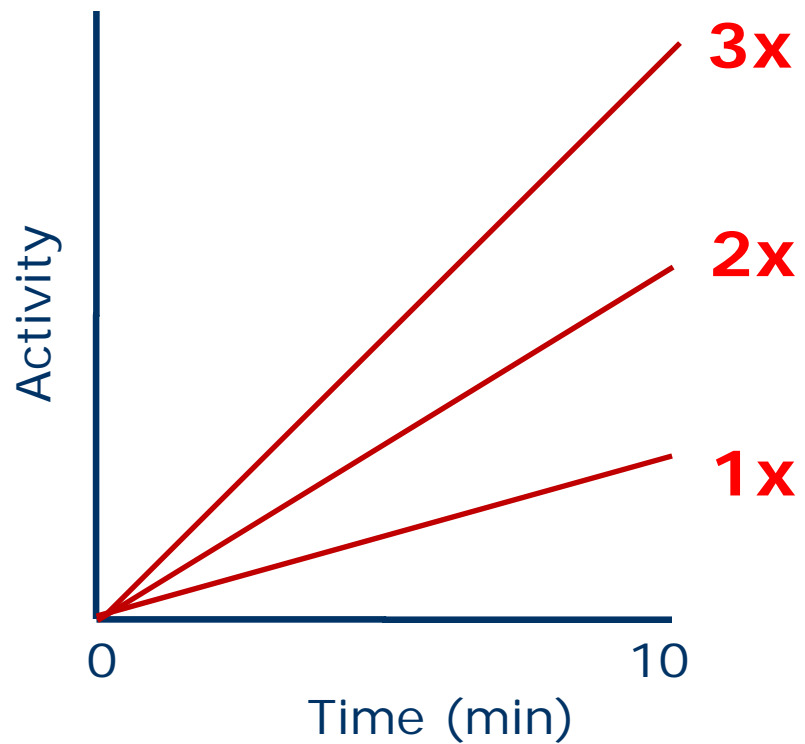
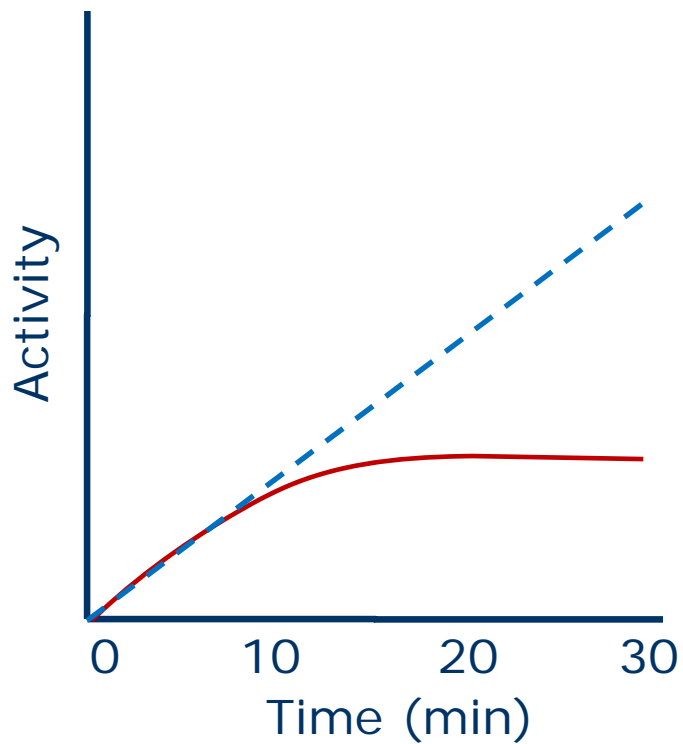
## Use of pH-specific buffer systems

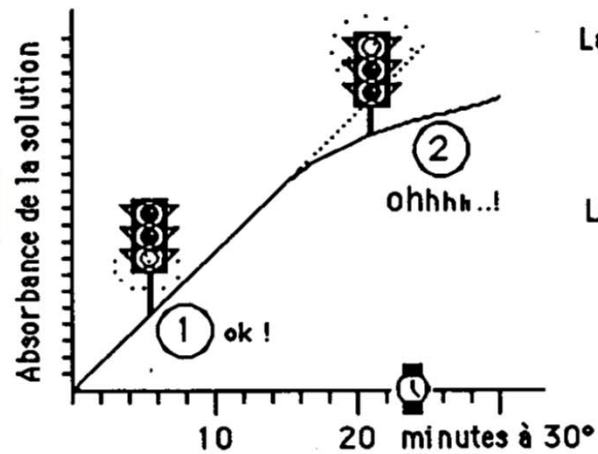
Useful pH Ranges of Selected Biological Buffers (25 °C, 0.1M)

Buffers	Useful pH Range	pKa (at 20)	pKa (at 25)	pKa (at 37)
MES	5.5–6.7	6.16	6.10	5.97
Bis-Tris	5.8–7.2	n/a	6.50	6.36
ADA	6.0–7.2	6.65	6.59	6.46
aces	6.1–7.5	6.88	6.78	6.54
PIPES	6.1–7.5	6.80	6.76	6.66
MOPSO	6.2–7.6	n/a	6.90	6.75
Bis-Tris Propane	6.3–9.5	n/a	6.8, 9.0	n/a
BES	6.4–7.8	7.17	7.09	6.90
MOPS	6.5–7.9	7.28	7.20	7.02
TES	6.8–8.2	7.50	7.40	7.16
HEPES	6.8–8.2	7.55	7.48	7.31
DIPSO	7.0–8.2	n/a	7.60	7.35
MOBS	6.9–8.3	n/a	7.60	n/a
TAPSO	7.0–8.2	n/a	7.60	7.39
Trizma	7.0–9.0	8.20	8.06	7.72
HEPPSO	7.1–8.5	n/a	7.80	6.66
POPSO	7.2–8.5	n/a	7.80	7.63
TEA	7.3–8.3	n/a	7.80	n/a
EPPS	7.3–8.7	n/a	8.00	n/a
Tricine	7.4–8.8	8.16	8.05	7.80
Gly-Gly	7.5–8.9	n/a	8.20	n/a
Bicine	7.6–9.0	8.35	8.26	8.04

note the effect of temperature on pKa

# Measurement of enzyme activity: Use initial rates!!!

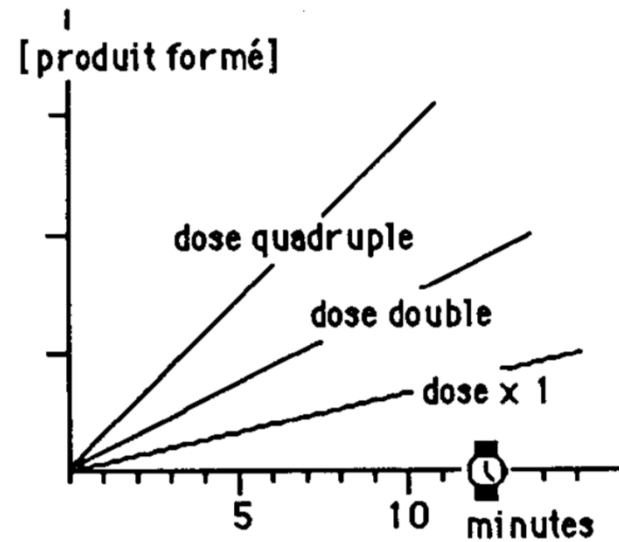




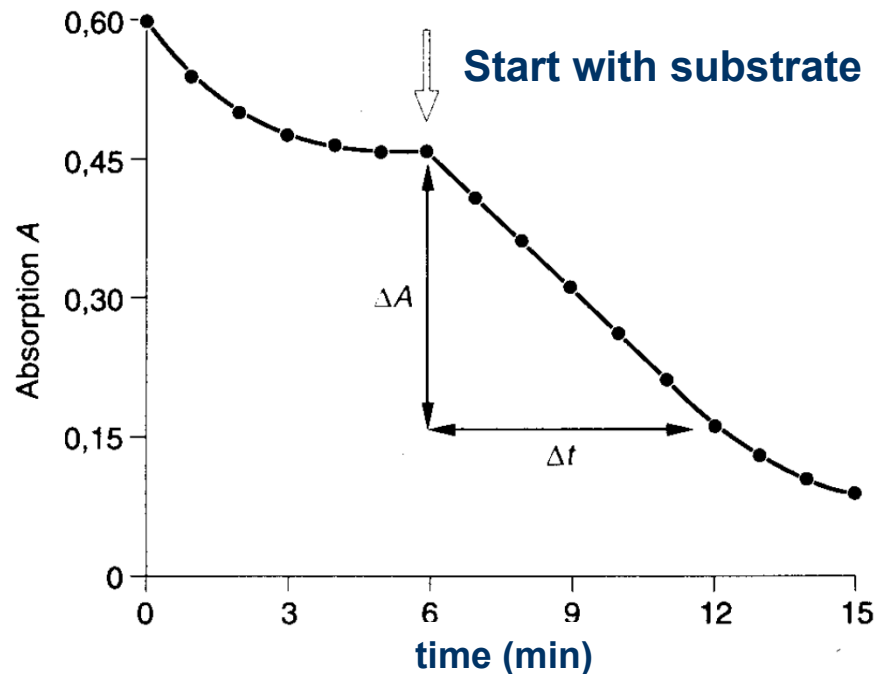
La phase 1, linéaire en fonction du temps, correspond à la **VITESSE INITIALE** de la réaction enzymatique.

La phase 2 peut être provoquée par:

- l'inactivation de l'enzyme.
- l'inhibition par le produit de la réaction ou la compétition avec la réaction inverse.
- l'épuisement du substrat



# Measurement of enzymatic product formation



**Beer-Lambert equation**

$$A = \log (I_0/I) = \epsilon \times c \times l$$

**Absorbance  $A = \epsilon \times c \times l$**

$\epsilon$  = extinction coefficient, (NADH at 340 nm,  $\epsilon = 6200 \text{ L mol}^{-1} \text{ cm}^{-1}$ )

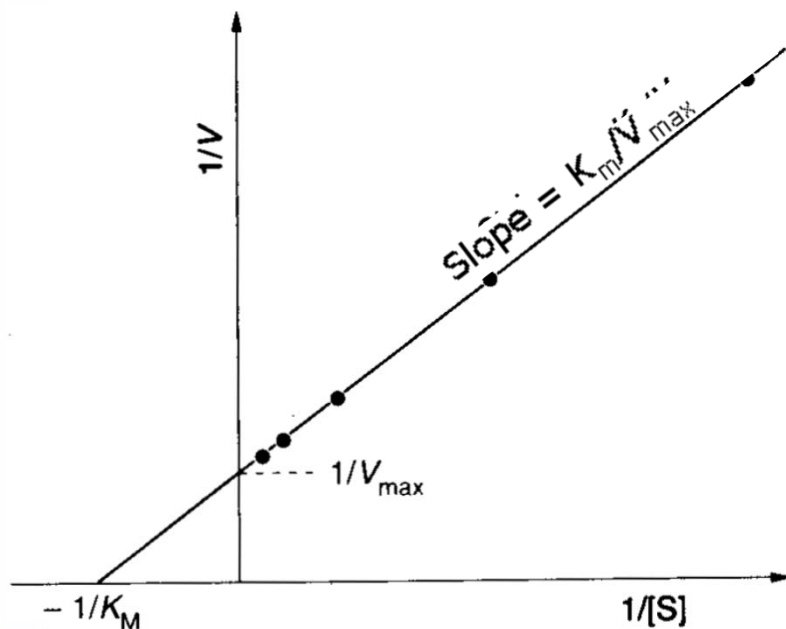
$c$  = concentration of NADH in mol / L

$l$  = thickness of the sample in cm

(usually 1.0 cm for standard sample cuvetts)

# Determination of kinetic parameters

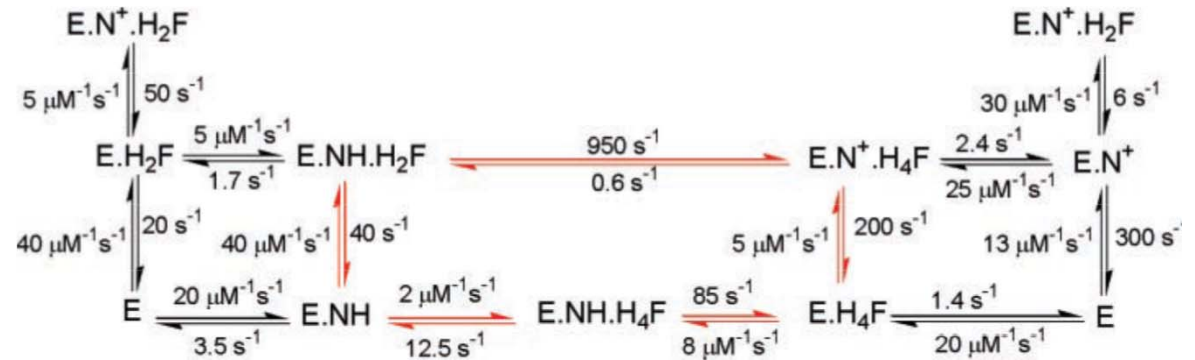
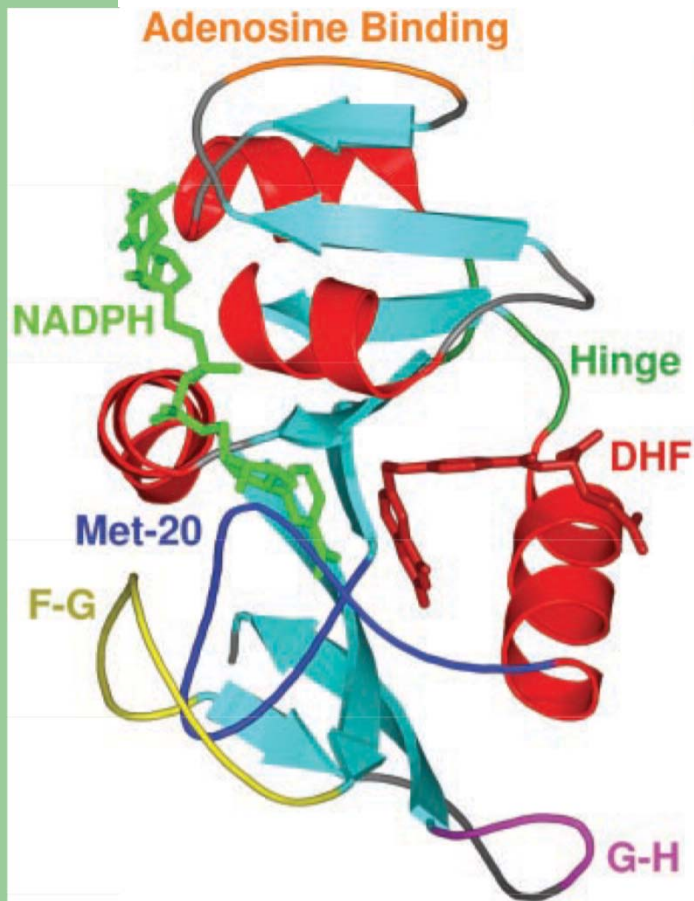
$V_{\max}$  determination:  $S \gg K_M \rightarrow V \approx V_{\max}$



Lineweaver Burke plot

$$\frac{1}{V} = \frac{K_M}{V_{\max}} \cdot \frac{1}{S} + \frac{1}{V_{\max}}$$

# Complexity of enzyme reactions: example DHFR



Why should we care?

# Complexity of enzyme reactions: Allosteric enzymes

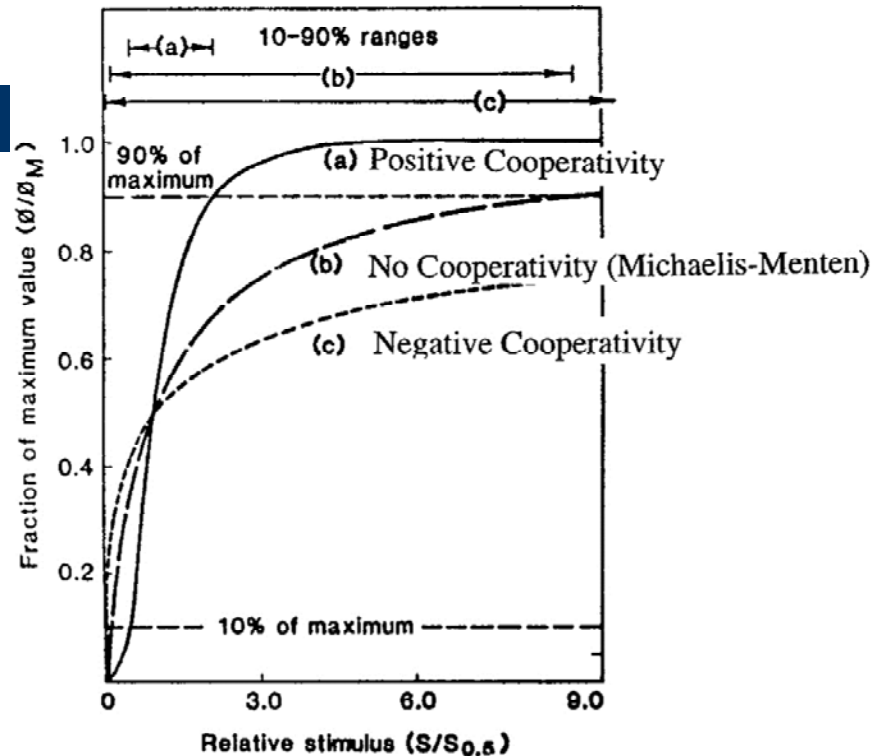


FIG. 1. The binding curve of a ligand to a protein with four identical subunits, each of which has one site for binding of a ligand (L). *a*, curve with positive cooperativity; *b*, curve with no cooperativity (Michaelis-Menten); and *c*, curve with negative cooperativity. Relative stimulus is stimulus,  $S$ , divided by stimulus when protein half-saturated,  $S_{0.5}$ .

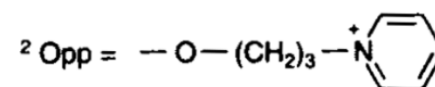
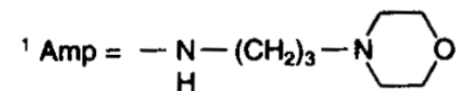
Why should we care?



## Application: Performance constant $K_{cat}/K_M$

Performance constant  $K_{cat}/K_M$  for the hydrolysis of various peptides by pepsin

Substrat	$k_{kat}$ ( $s^{-1}$ )	$K_M$ ( $mmol \cdot L^{-1}$ )	$k_{kat}/K_M$ ( $L \cdot mol^{-1} \cdot s^{-1}$ )
Z-Ala-Phe(NO <sub>2</sub> )-Apm <sup>1</sup>	0,002	1,46	1,4
Z-Phe(NO <sub>2</sub> )-Val-Apm <sup>1</sup>	0,010	0,78	13
Z-Phe(NO <sub>2</sub> )-Phe-Apm <sup>1</sup>	0,052	0,74	70
H-Gly-Gly-Phe-Phe-Apm <sup>1</sup>	0,950	3,90	244
Z-Gly-Pro-Phe-Phe-Opp <sup>2</sup>	0,056	0,14	400
Z-Val-Phe(NO <sub>2</sub> )-Phe-Apm <sup>1</sup>	0,310	0,17	1824
Z-Ala-Ala-Phe(NO <sub>2</sub> )-Phe-Apm <sup>1</sup>	43,800	1,51	29000
Z-Ala-Gly-Phe-Phe-Opp <sup>2</sup>	145,000	0,25	$5,8 \cdot 10^5$
Z-Ala-Ala-Phe-Phe-Opp <sup>2</sup>	282,000	0,04	$7,05 \cdot 10^6$



Application: Inhibitor studies, Mechanism?

Performance vs specificity constant?

# Different types of enzyme assays

**Photometric enzyme assays:** (chromogenic substrates)

Dehydrogenases

Nicotine Adenine Dinucleotide (direct/indirect)

$\text{NAD}^+ + 2\text{H}^+ + 2\text{e}^- = \text{NADH} + \text{H}^+ \rightarrow$  light absorption at 340 nm

Proteases/ $\beta$ -Galactosidases: *p*-Nitroanilin/*o*-Nitrophenol

**Fluorometric enzyme Assays:** Dehydrogenases

**Luminometric enzyme assays:** ATPase

**HPLC – product formation**

**O<sub>2</sub> production/consumption (electrode)**

**Many others...**

# Enzyme Assays in the lab...

## Desirable Enzyme Properties(for assaying purposes..)

Easy to purify

Completely stable

Activity proportional to concentration over wide range

Can be easily assayed

Not subject to interference by other compounds

Can be frozen for storage

Is resistant to proteases

Others?

# Enzyme Assays in the lab...

## Perfect Assay

- Substrates are inexpensive
- Substrates are soluble
- Substrate saturates enzyme (is in great excess during assay)
- Is very sensitive
- Substrates are stable
- Product is stable
- Others?

## Enzyme Assays in the lab...

In practice, our assumptions regarding enzyme assays (linearity, stability) may not always be easy to satisfy.

Therefore we must perform linearity checks (use a standard curve...) and employ controls...

# Measurement of protein concentration

Must measure protein to determine activity  
Specific activity = Total Activity/Protein Concentration

**N total:** (Kjeldahl)

**Biuret:**  $\text{Cu}^{2+}$ /tartrate complex

**Lowry:** Biuret with Folin reagent (Hg-Phenols)

**UV:** peptide bond -  $A_{215}$ , aromatic (F, Y, W)  $A_{280}$

**Bradford:** Binding of Coomassie  $G_{250}$  to  $-\text{NH}_2$  and R  $\rightarrow$  OD 595 nm  
(the method we use...)