Techniques in Molecular Genetics

Spectroscopy and Enzyme Assays

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)

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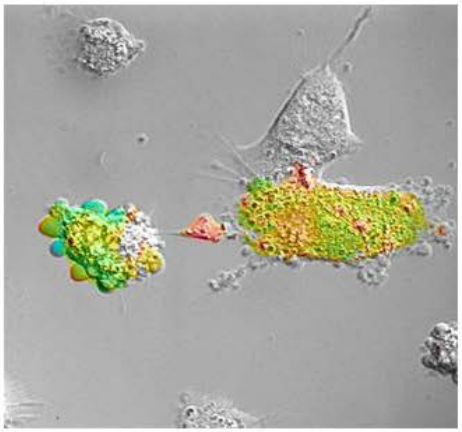
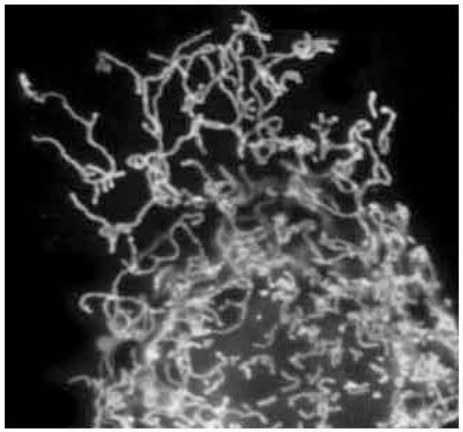
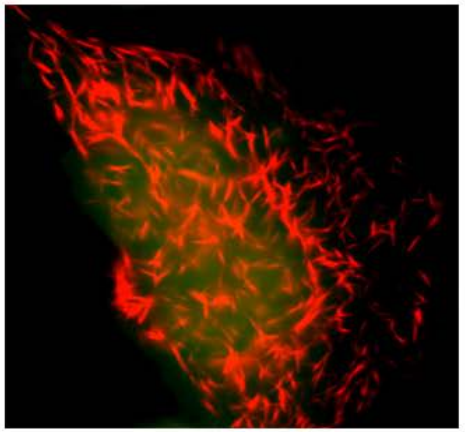
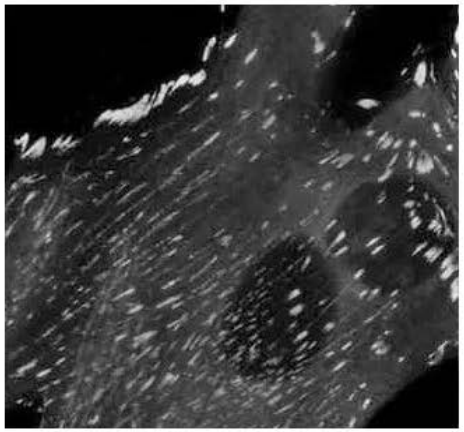
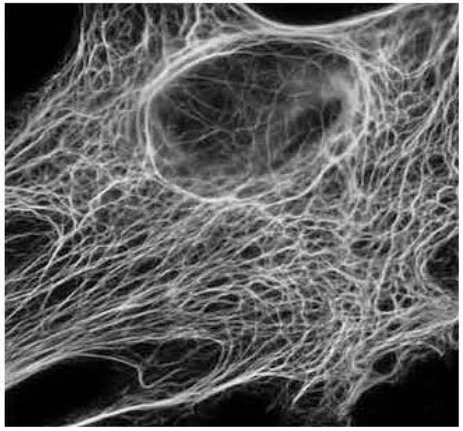
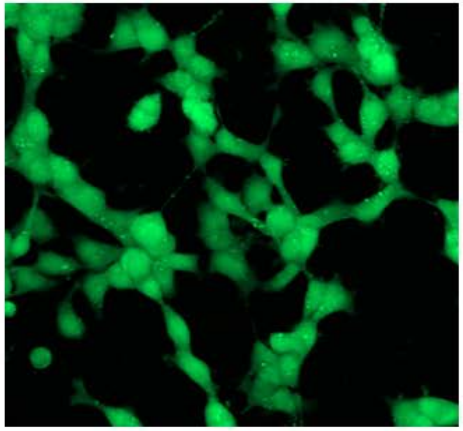
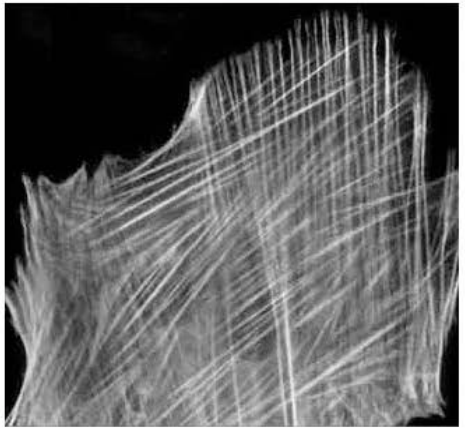
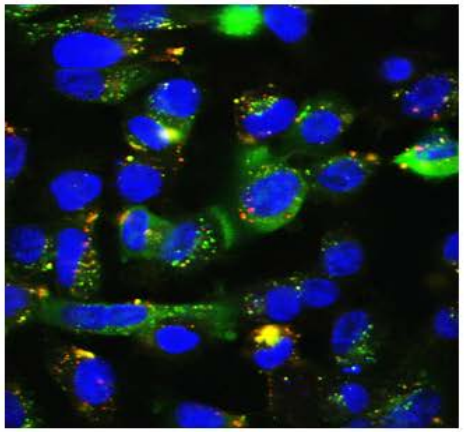
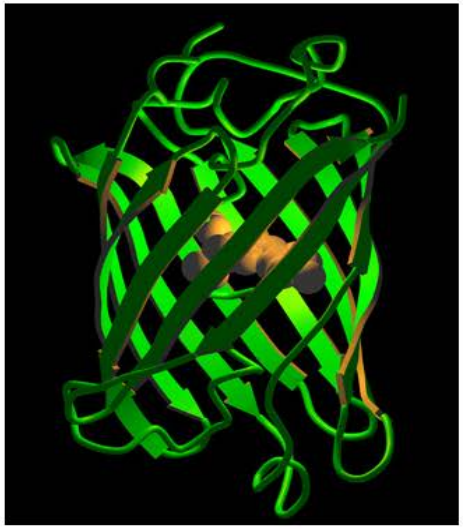
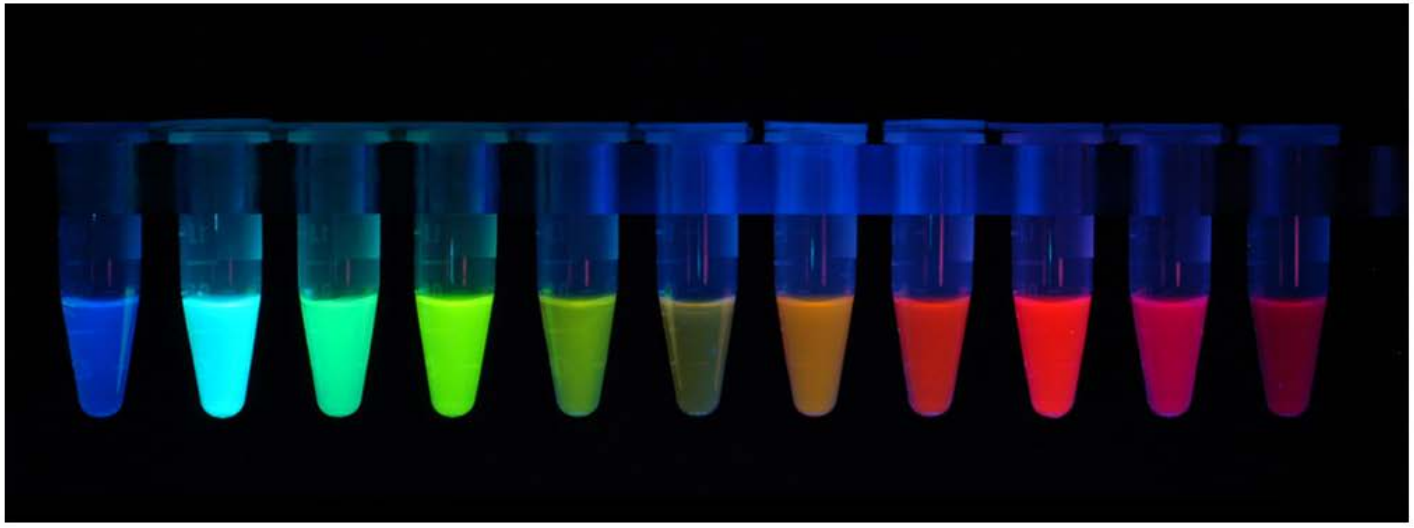
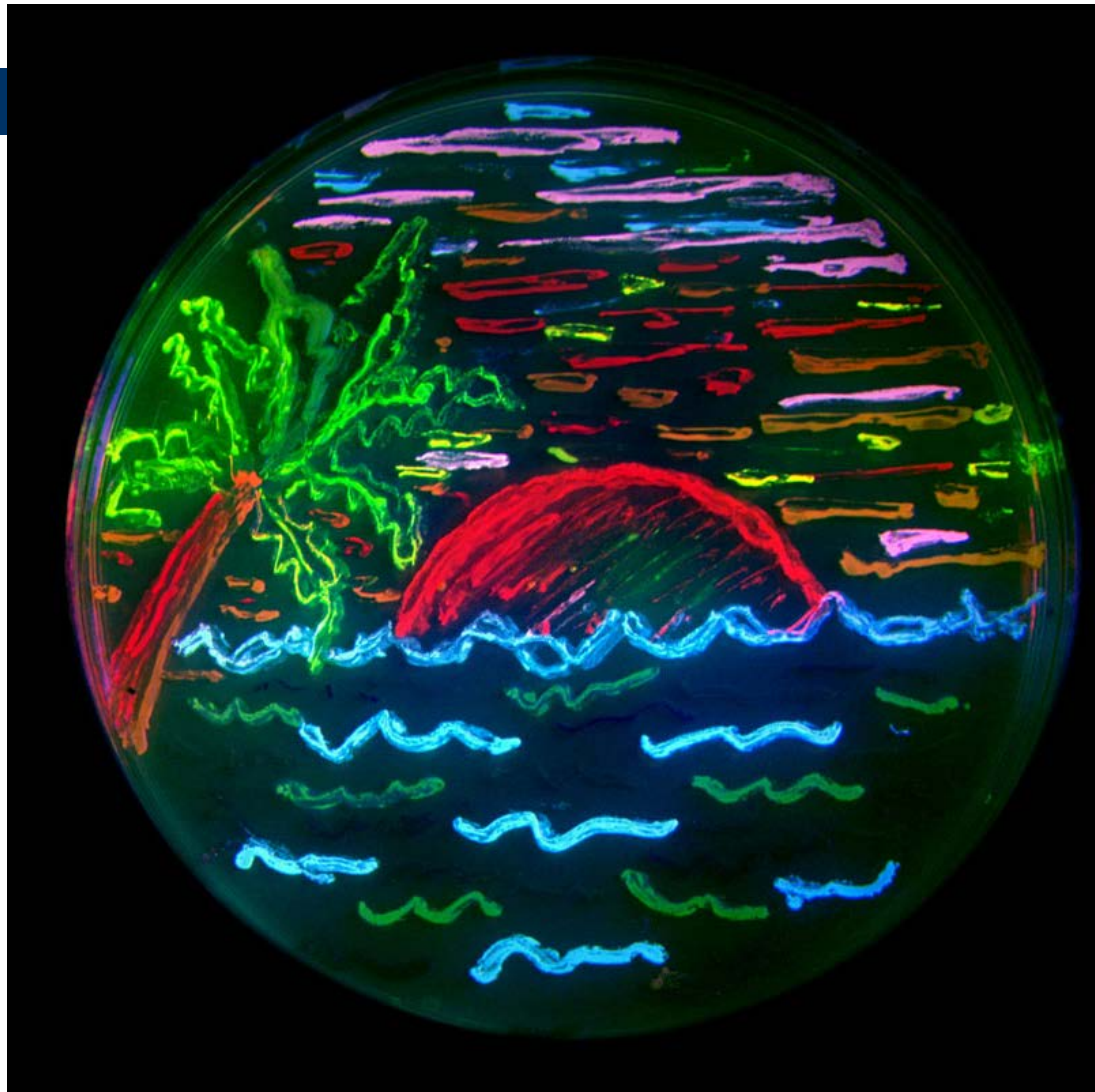











Also can be used for practicing streaking technique…



Abbe Limit broken! (after 120 years)

Abbe limit (d) = λ / 2 x NA

Ernst Abbe
(1840-1905)

News and Views

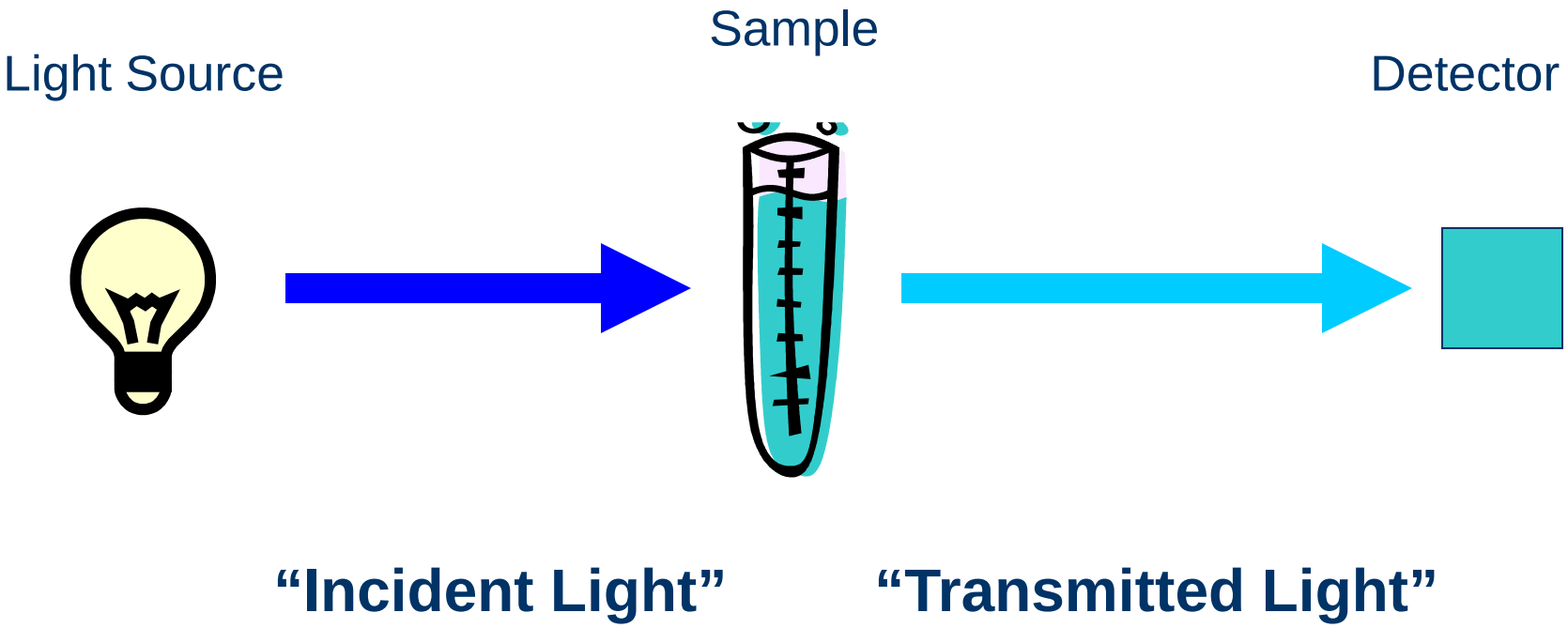
Nature **417**, 806-807 (20 June 2002) | doi:10.1038/417806a

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 (ε) for the dissolved substance

$$T = 10^{\varepsilon c L} \text{ or } \quad A_\lambda = \varepsilon\, c\, L,$$

ε – *Molar Extinction Coefficient*

A_λ – 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 k_{non}, (s^{-1})	Enzymatic Rate k_{cat} (s^{-1})	Relative Rate k_{cat}/k_{non}
Cyclophilin	2.8×10^{-2}	1.3×10^{4}	**4.6×10^{5}**
Carbonic anhydrase	1.3×10^{-1}	1.0×10^{5}	**7.7×10^{6}**
Chymotrypsin	4.0×10^{-9}	4.0×10^{-2}	**1.0×10^{7}**
Triosephosphate isomerase	6.0×10^{-7}	2.0×10^{3}	**3.0×10^{9}**
Fumarase	2.0×10^{-8}	2.0×10^{3}	**1.0×10^{11}**
Adenosine deaminase	1.8×10^{10}	370	**2.1×10^{12}**
Urease	3.0×10^{-10}	3.0 x 10'	**1.0×10^{14}**
Alkaline phosphatase	1.0×10^{-15}	1.0×10^{2}	**1.0×10^{17}**

Source: various

Basics of enzyme kinetics: unit definitions

Rate of reaction = moles of substrate consumed/product formed (mol L^{-1} s^{-1})

Enzyme activity = moles converted per unit time = rate x volume

SI unit **katal** 1 katal = 1 mol s^{-1}

unit (U) 1 unit = µmol min^{-1}

Specific activity = moles converted per unit time per unit mass of enzyme → enzyme purification

katal kg^{-1} / µmol mg^{-1} min^{-1} / µmol $µg^{-1}min^{-1}$

Turnover number = moles converted per unit time per mole of enzyme (s^{-1}) K_{cat}

Enzyme purification and specific activity

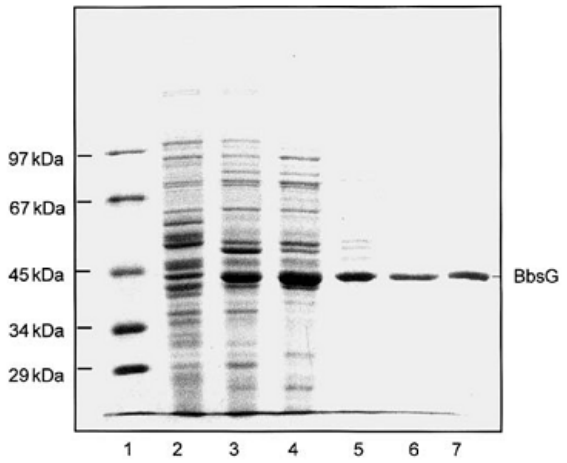


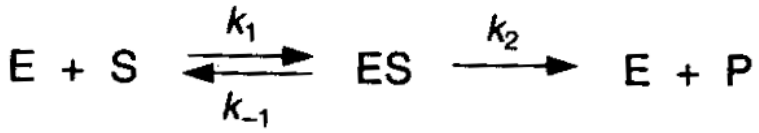
Fig. 2 SDS-PAGE of protein fractions containing (*R*)-benzylsuccinyl-CoA dehydrogenase (*BbsG*) activity. Coomassie-blue-stained SDS-polyacrylamide gel (10% polyacrylamide; w/v). *Lanes*: *1* Marker, *2* control extract of *Escherichia coli* lacking BCDH, *3 E. coli* extract containing recombinant BCDH, *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 µmol benzylsuccinyl-CoA oxidised min^{-1}

Fraction	Volume (ml)	Protein (mg)	Activity (U)	Specific activity (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

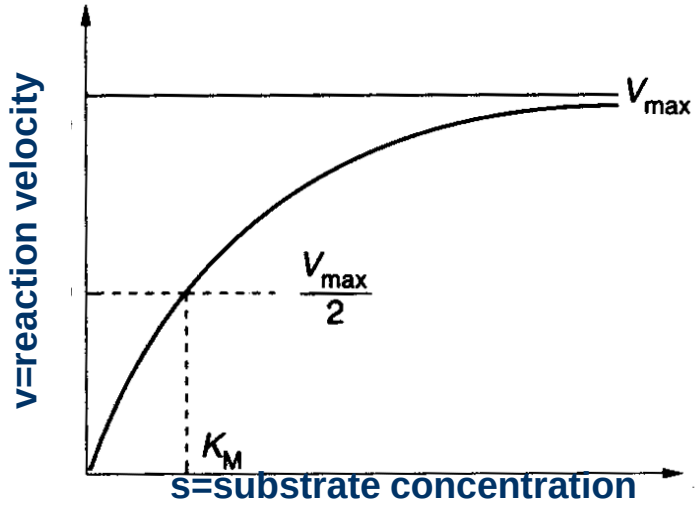
Source: various

Basics of enzyme kinetics: Michaelis-Menten



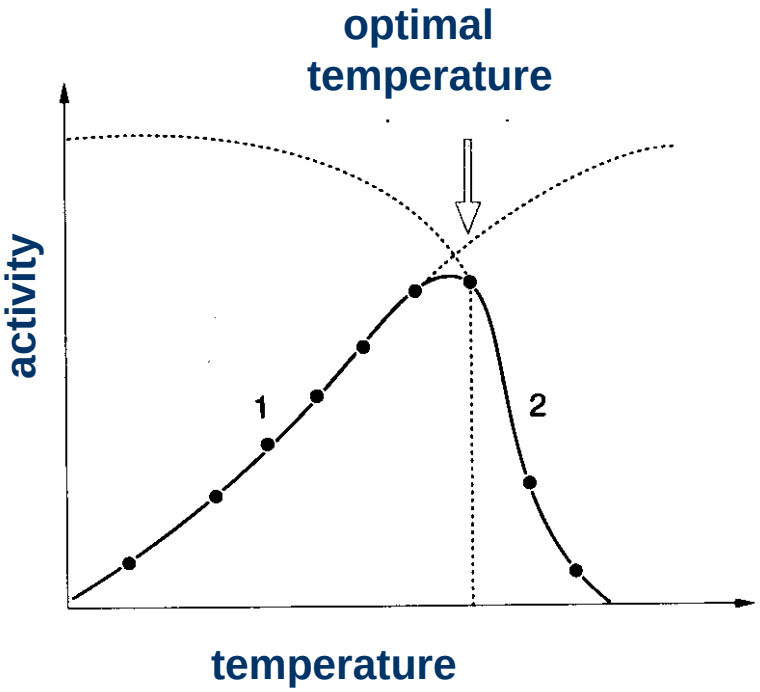
Michaelis-Menten

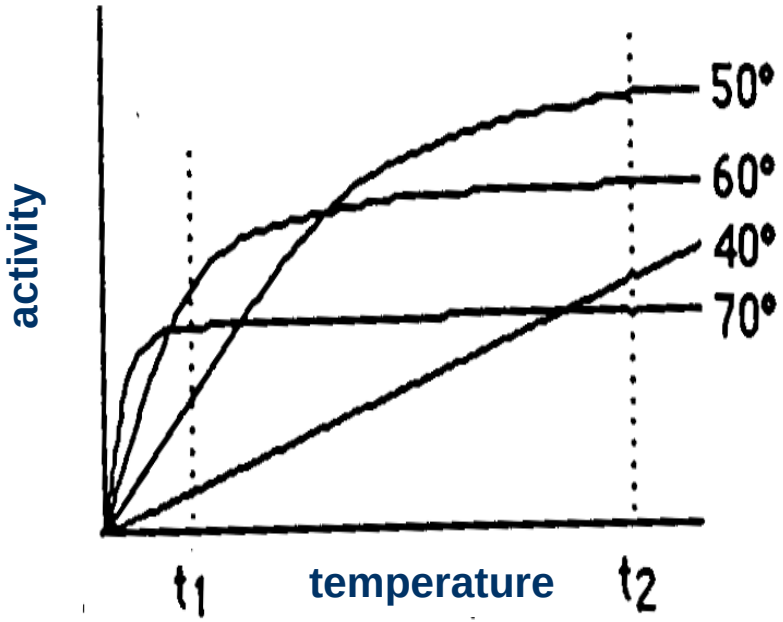
$$V = \frac{V_{max} \cdot [S]}{K_M + [S]}$$



K_M => $V_{max}/2$

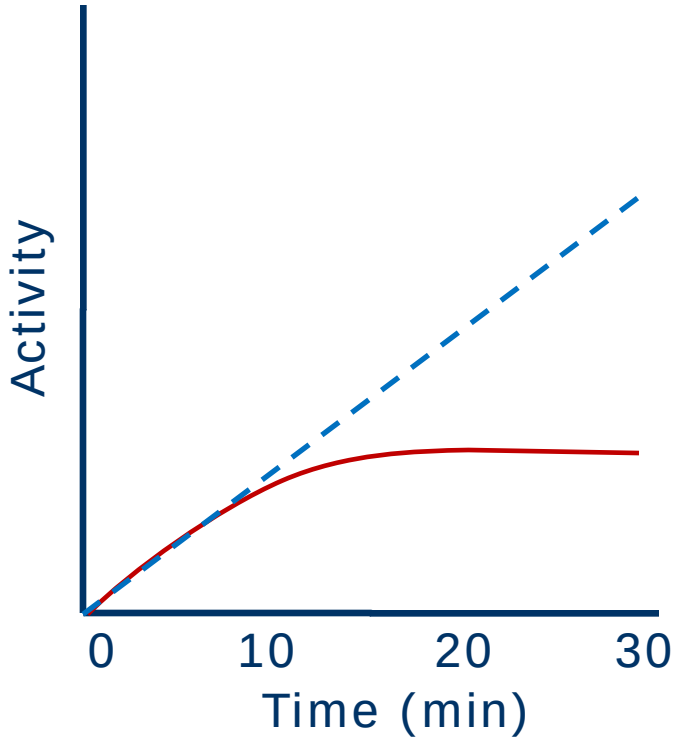
Factors affecting enzyme activities: Temperature

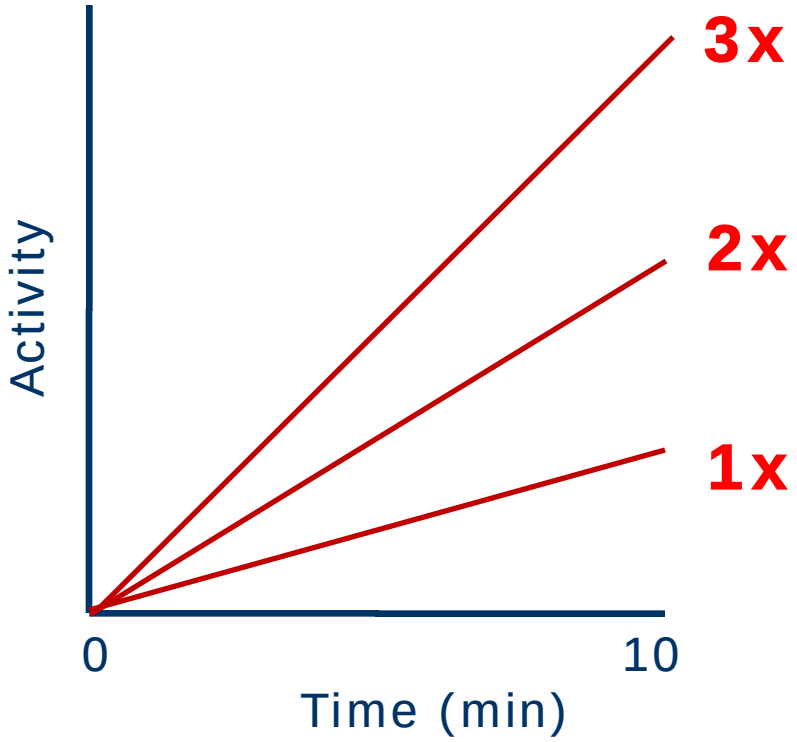




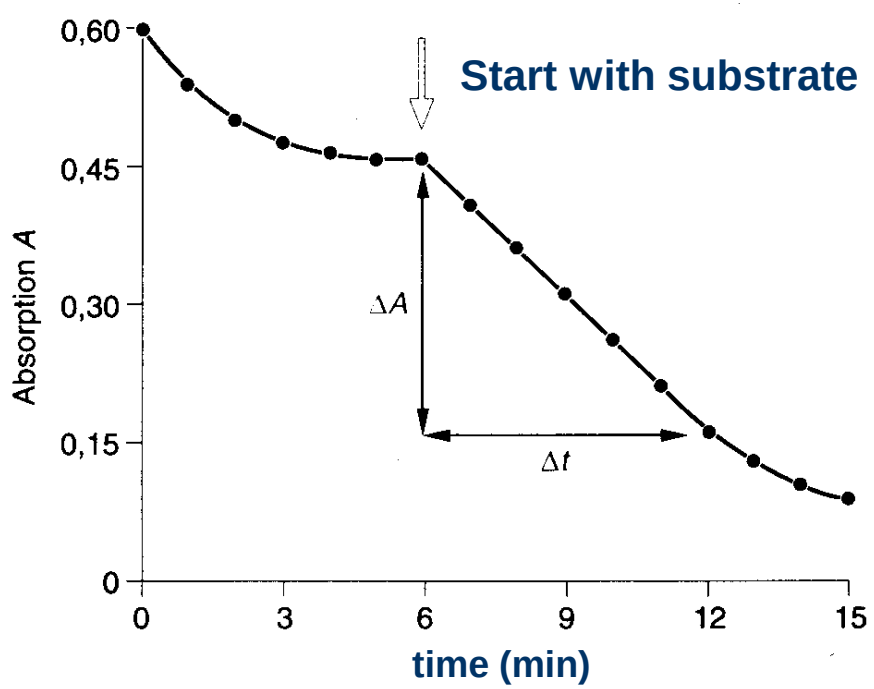
Which enzyme is most thermostable?

Measurement of enzyme activity: Use initial rates!!!





Measurement of enzymatic product formation



Beer-Lambert equation

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

Absorbance $A = \varepsilon \times c \times l$

ε = extinction coefficient, (NADH at 340 nm, ε = 6200 L mol^{-1} cm^{-1})

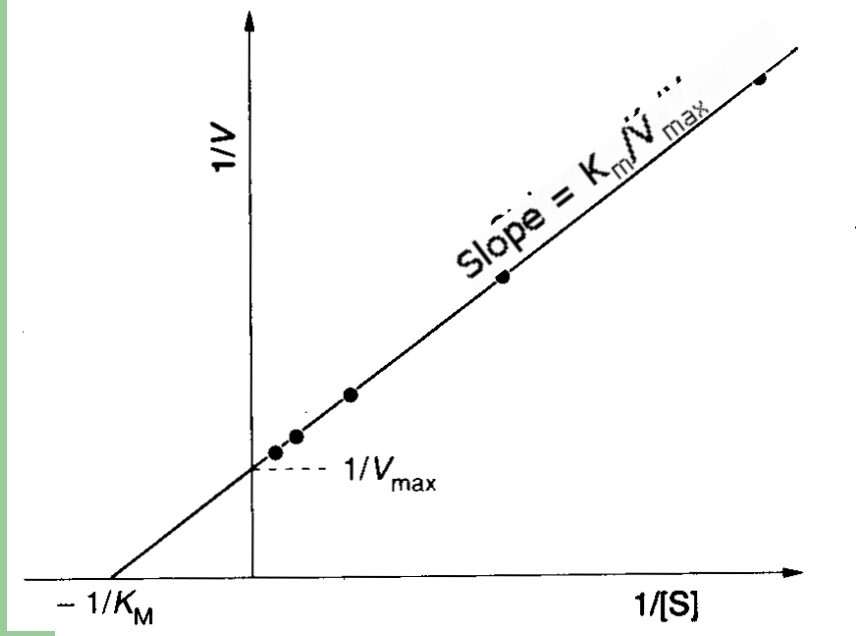
c = concentration of NADH in mol / L

l = thickness of the sample in cm

(usually 1.0 cm for standard sample cuvets)

Determination of kinetic parameters

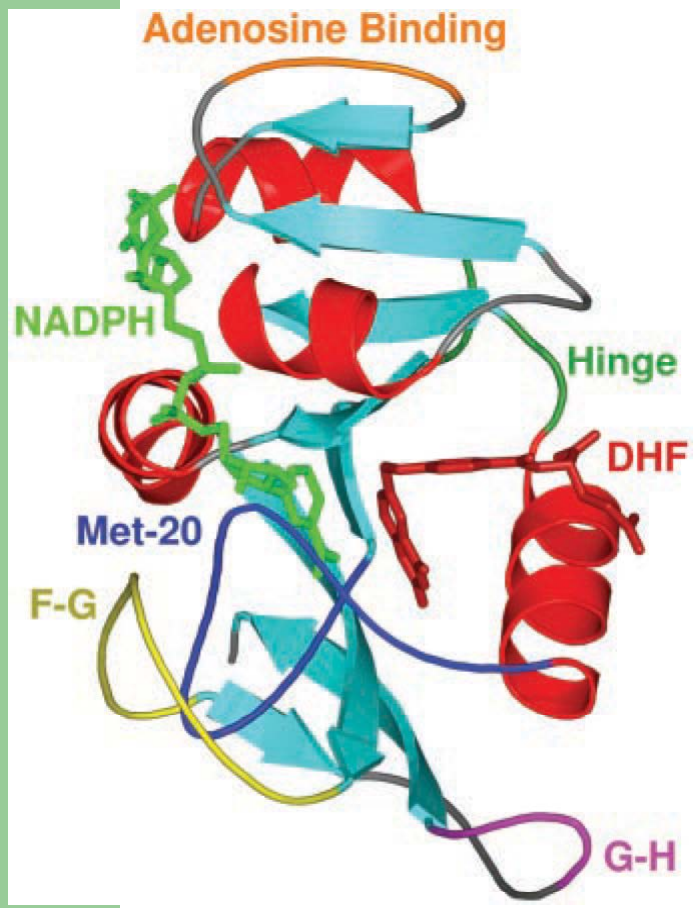
V_{max} determination: S >> K_M → V ≈ V_{max}

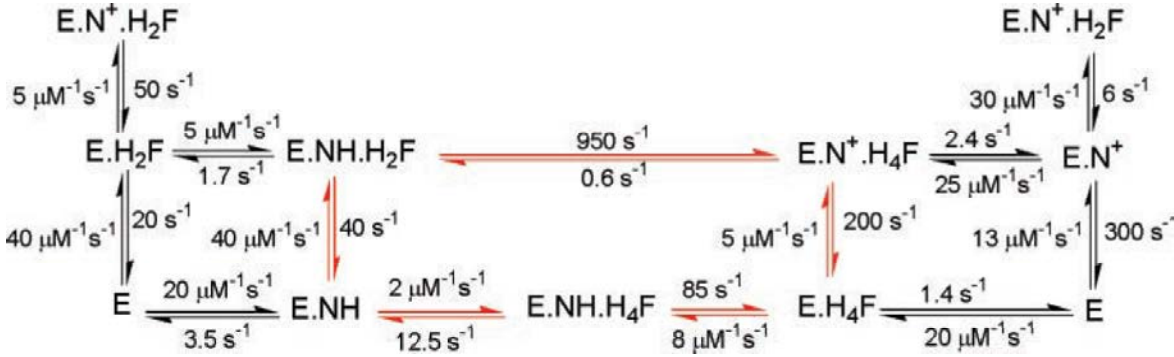


Lineweaver Burke plot

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

Complexity of enzyme reactions: example DHFR





Why should we care?

Complexity of enzyme reactions: Allosteric enzymes

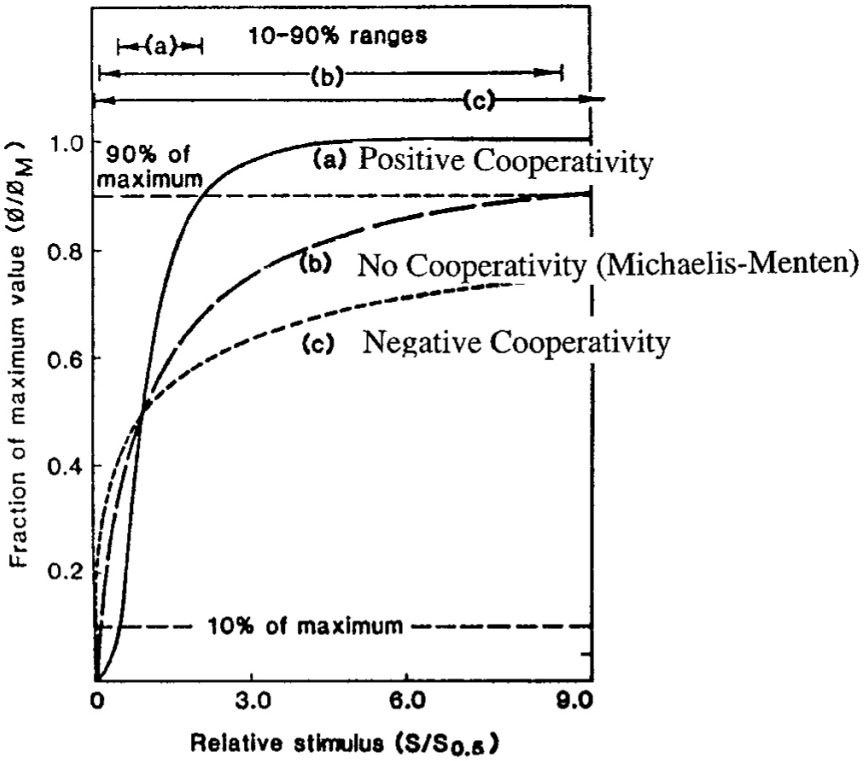


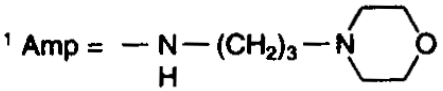
FIG. 1. **The binding curve of a ligand to a protein with four identical subunits, each of which has one site for binding of a ligand (L).** *a*, *curve* with positive cooperativity; *b*, *curve* with no cooperativity (Michaelis-Menten); and *c*, *curve* with negative cooperativity. Relative stimulus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

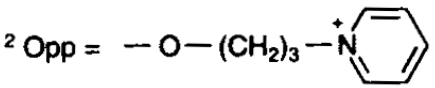
Why should we care?

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 · L^{-1})	k_{kat}/K_M (L · mol^{-1} · s^{-1})
Z-Ala-Phe(NO_2)-Apm[1]	0,002	1,46	1,4
Z-Phe(NO_2)-Val-Apm[1]	0,010	0,78	13
Z-Phe(NO_2)-Phe-Apm[1]	0,052	0,74	70
H-Gly-Gly-Phe-Phe-Apm[1]	0,950	3,90	244
Z-Gly-Pro-Phe-Phe-Opp[2]	0,056	0,14	400
Z-Val-Phe(NO_2)-Phe-Apm[1]	0,310	0,17	1824
Z-Ala-Ala-Phe(NO_2)-Phe-Apm[1]	43,800	1,51	29000
Z-Ala-Gly-Phe-Phe-Opp[2]	145,000	0,25	$5{,}8 \cdot 10^5$
Z-Ala-Ala-Phe-Phe-Opp[2]	282,000	0,04	$7{,}05 \cdot 10^6$

[1] Amp = 

[2] Opp = 

Application: Inhibitor studies, Mechanism?

Performance vs specificity constant?

Different types of enzyme assays

Photometric enzyme assays: (chromogenic substrates)

Dehydrogenases

Nicotine Adenine Dinucleotide (direct/indirect)

$NAD^+ + 2H^+ + 2e^- = NADH + H^+ \rightarrow$ light absorbtion at 340 nm

Proteases/β-Galactosidases: *p*-Nitroanilin/*o*-Nitrophenol

Fluorometric enzyme Assays: Dehydrogenases

Luminometric enzyme assays: ATPase

HPLC – product formation

O_2 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: 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 $-NH_2$ and R → OD 595 nm
(the method we use...)