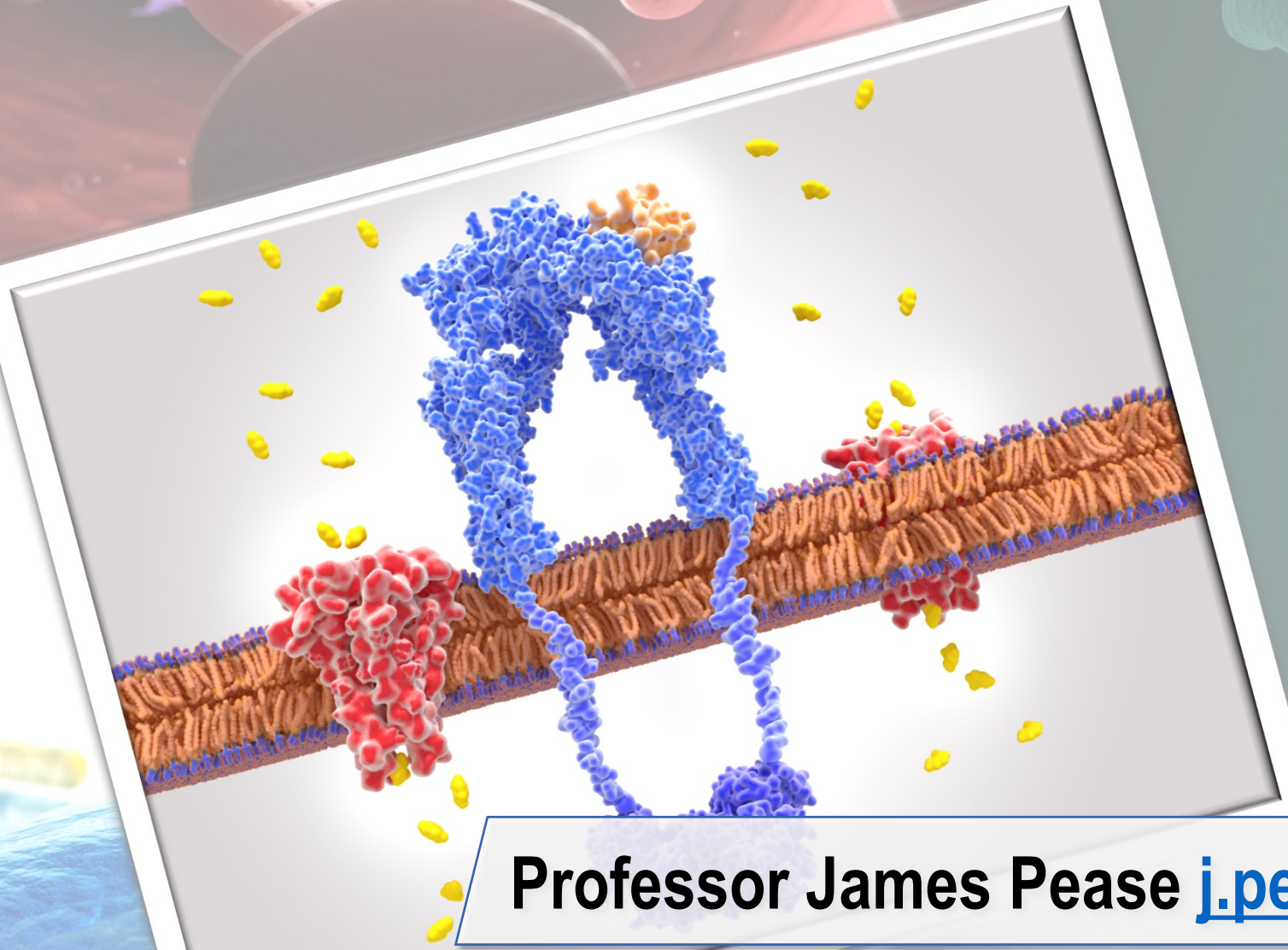
IMPERIAL

Enzyme Kinetics



Professor James Pease j.pease@imperial .ac.uk

Session plan

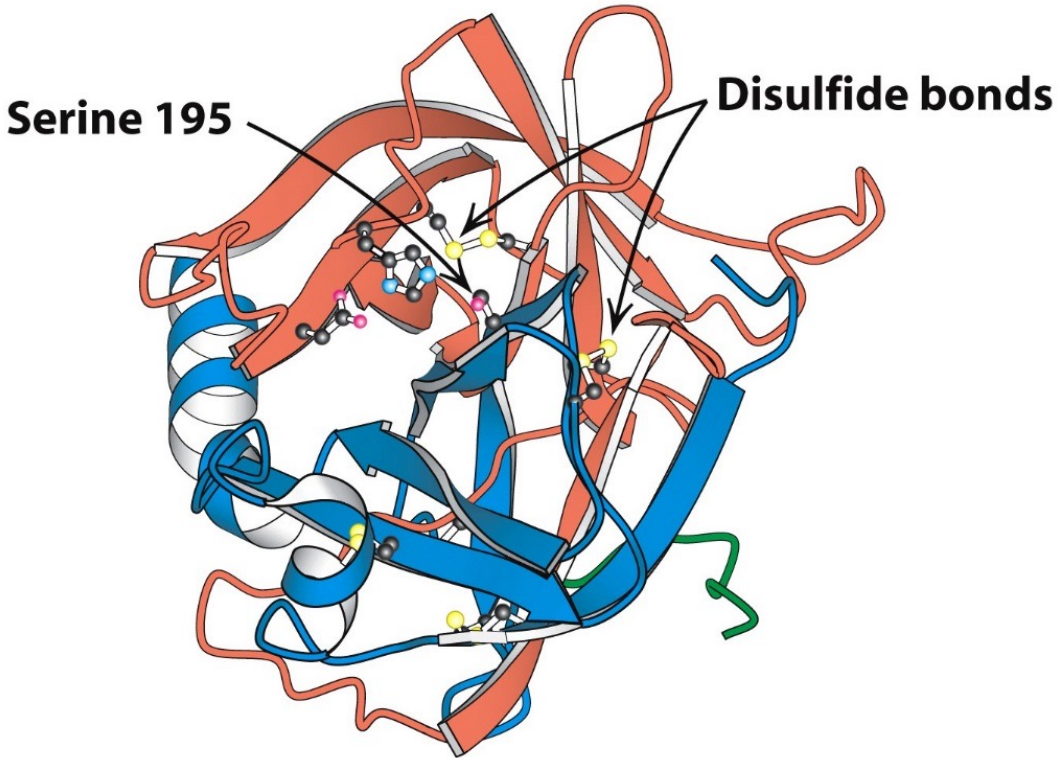
Part 1 – “wet”

- Study the cleavage of a peptide by chymotrypsin
- Use an appropriate wavelength to monitor the rate of product formation.
- By altering substrate concentration, gain valuable information regarding the enzyme activity.

Part 2 – “dry”

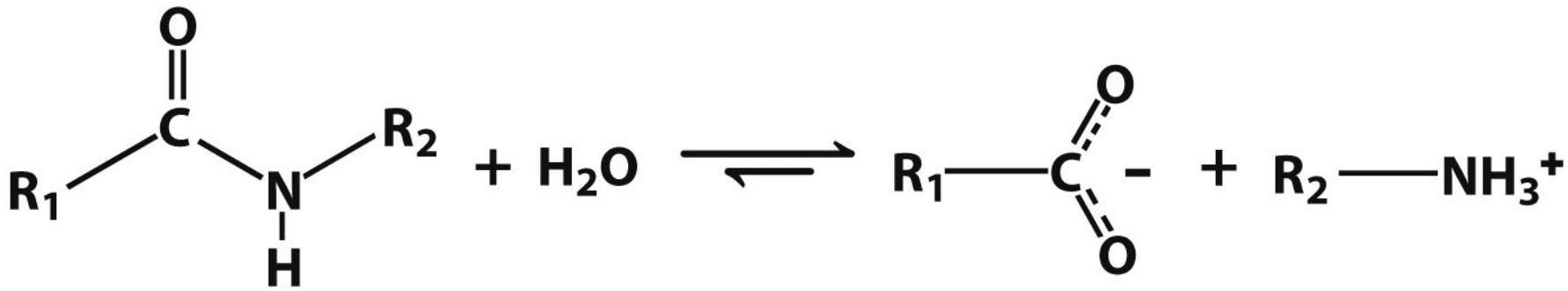
- Generate Graph 2 – a plot of absorbance versus time for several substrate concentrations.
- Convert rate of change in absorbance into concentration of product
- Generate Graph 2 (Lineweaver burk plot)
- Calculate K_M and V_{max}. Enter into Qualtrics

Chymotrypsin – a model protease



- Serine protease found in the digestive system of mammals.
- Arranged in three peptide chains (A, B and C) linked by disulphide bridges
- Proteases also key for regulation other processes:
- Protein maturation (e.g. removal of signal peptides)
- Degradation of ECM by migrating cells
- General protein turnover.

Chymotrypsin cleaves certain peptide bonds



Chymotrypsin has specificity for bulky hydrophobic side chains

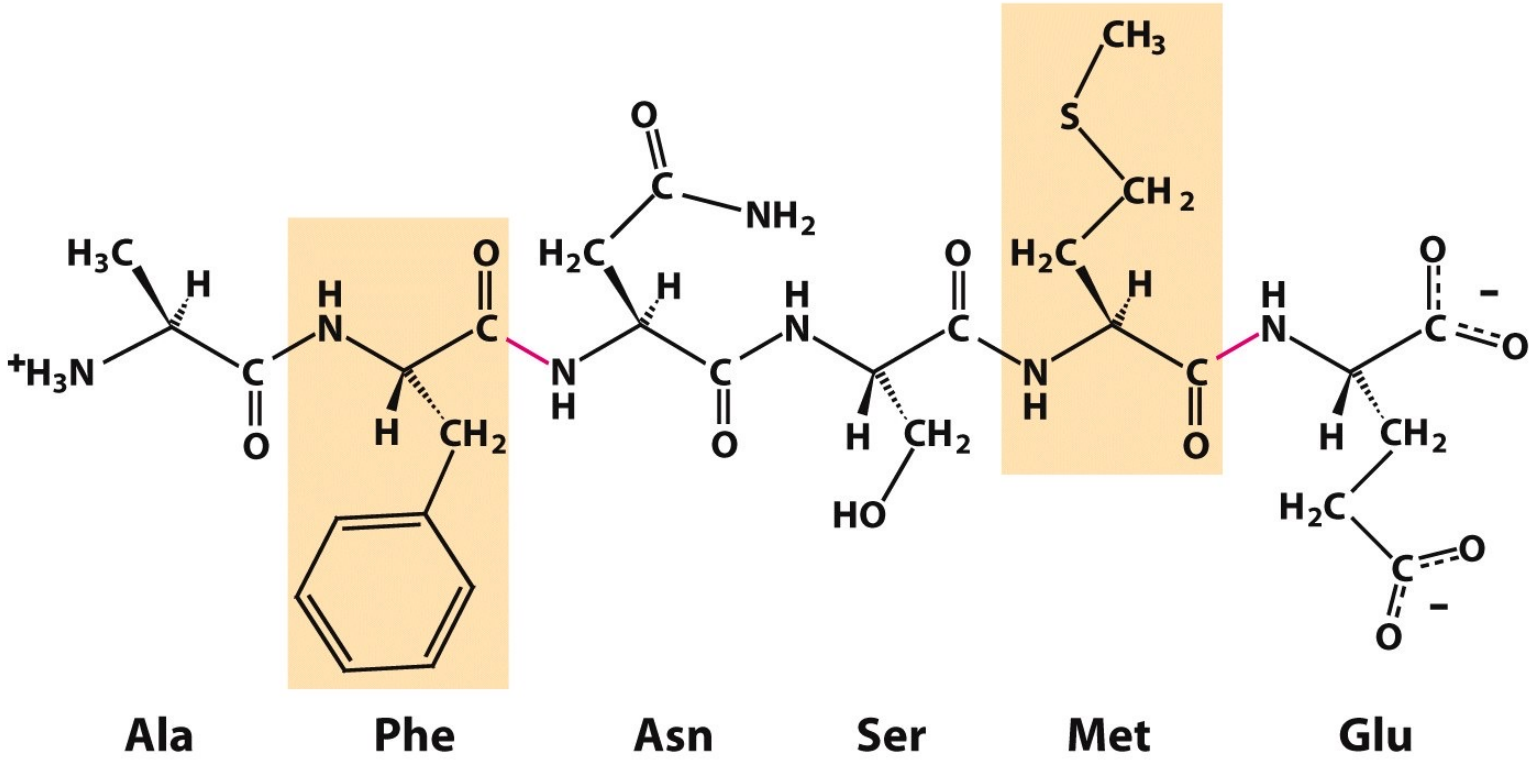
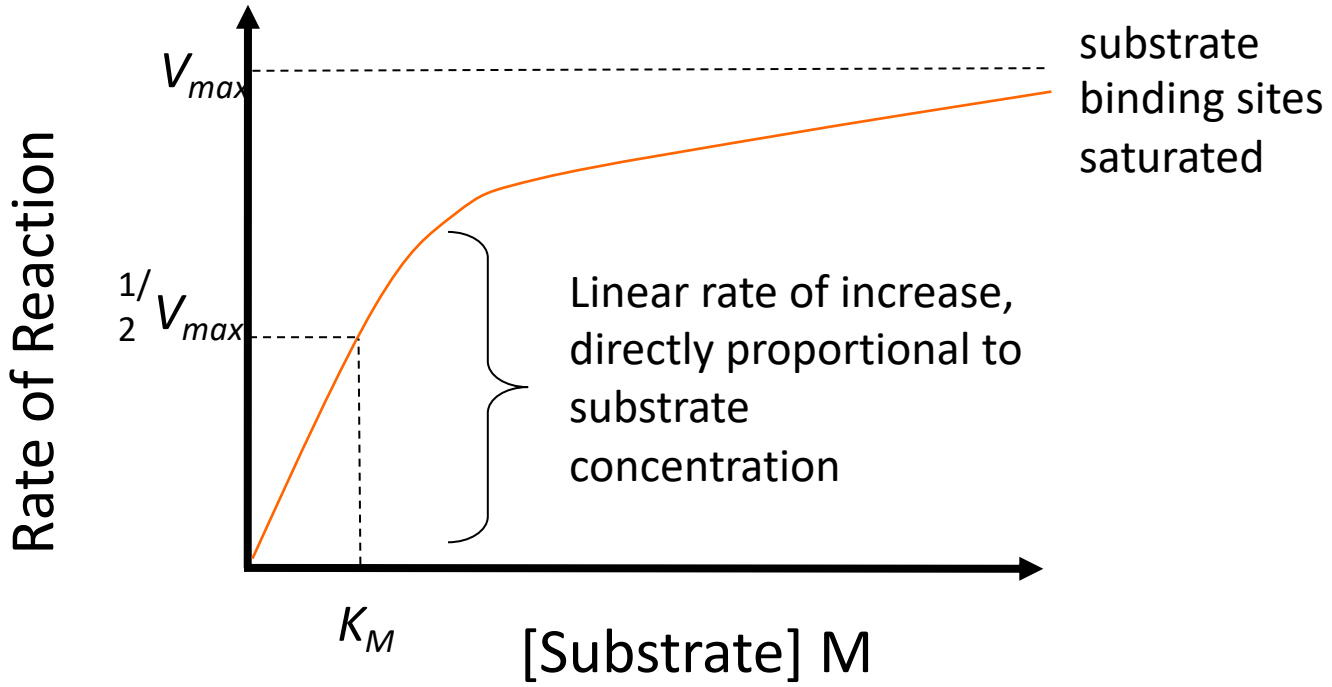


Figure 9.1
***Biochemistry*, Seventh Edition**

- The bond in cyan is cleaved, between the phenylalanine (Phe) and asparagine (Asn).

Enzyme kinetics – parameter definitions



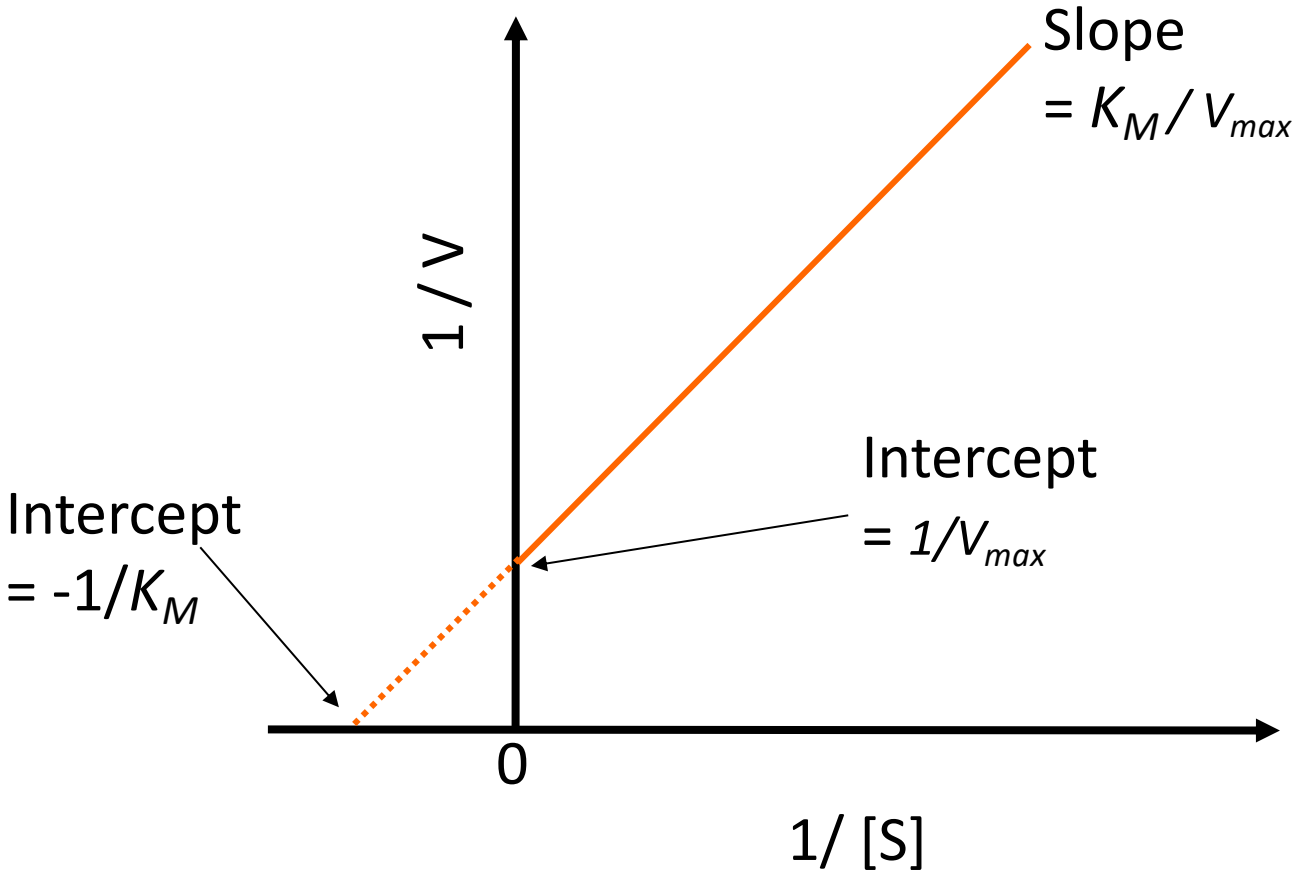
K_M is known as the Michaelis Constant and is defined as the concentration of substrate at which a particular enzyme works at half its maximal velocity.

Biochemically, the K_M value is useful as a means of comparing the strength of Enzyme-Substrate complexes.

Generally a low K_M indicates tight binding of a substrate to an enzyme.

Conversely, a high K_M is indicative of weak binding.

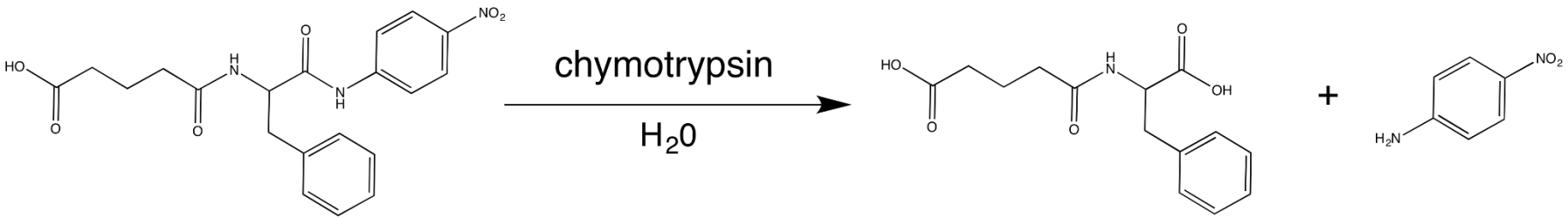
Lineweaver-Burk plot



A double-reciprocal plot of 1 / V against 1 / [S]

Intercepts give us K_M and V_{max}.

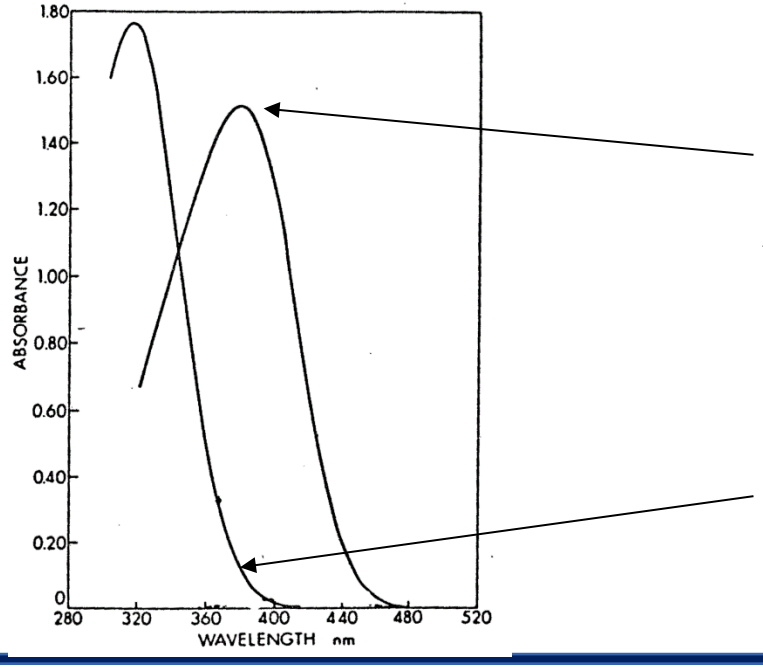
Reaction scheme and detection



N-Glutaryl-L-phenylalanine *p*-nitroanilide (GPNA)

N-glutaryl-L-phenyl alanine

p-nitroaniline



p-nitroaniline (yellow)

GPNA

- Absorbance at 410 nm allows us to monitor production formation.

Protocol outline

- 2 students per spectrophotometer
- Measure the production of *p*-nitroaniline at 410 nm at a variety of GPNA concentrations and determine the rate of reaction (**Graph 1**).
- Construct a Lineweaver-Burk plot (**Graph 2**). From this:
- Calculate V_{max}
- Calculate K_M
- Takes 1 hour to generate a dataset. 1 hour to plot the graphs.

Protocol

You are provided with:

20ml of Reaction buffer, pH 7.8
5ml of a stock solution of 20 mmM GPNA (labelled S).
0.4ml of chymotrypsin at 20mg/ml (labelled E)

Ensure that the spectrophotometer is set to 410 nm.

You will pipette varying amounts of the stock GPNA solution and the reaction buffer into a 3ml cuvette to create a range of substrate concentrations.

Mix & Calibrate (press CAL button).

Add Enzyme (Mix again) and start recording A_{410} for 3 minutes.

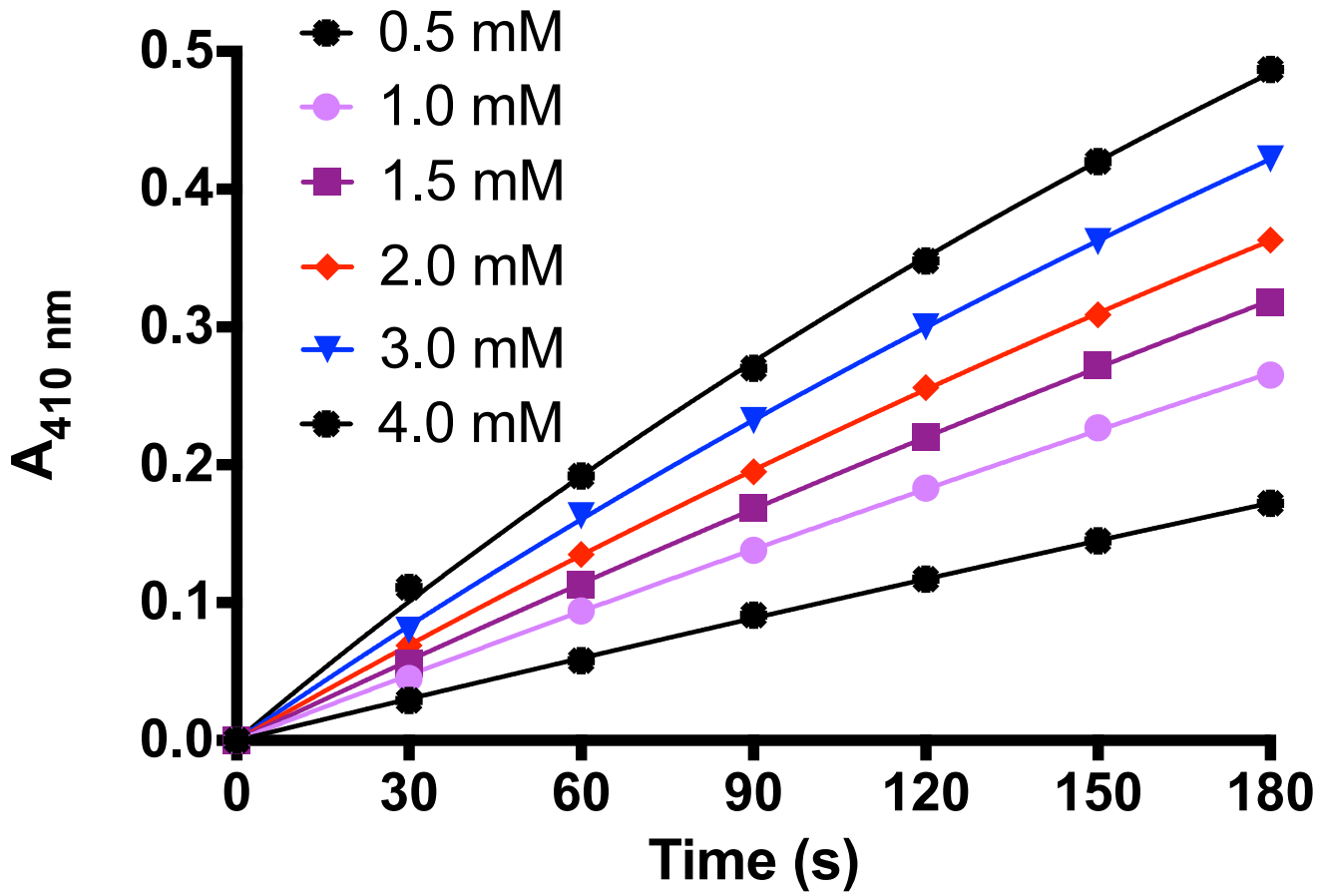
Reaction volumes

Volume of Stock GPNA (ml)	Volume of Buffer (ml)	Volume of enzyme (ml)	Total Volume in Cuvette (ml)	Final [GPNA] in cuvette (mM)
0.600	2.350 (3X 0.783)	0.050	3.000	4.0
0.450	2.500 (3x 0.833)	0.050	3.000	3.0
0.300	2.650 (3 x 0.883)	0.050	3.000	2.0
0.225	2.725 (3 x 0.908)	0.050	3.000	1.5
0.150	2.800 (3 X 0.933)	0.050	3.000	1.0
0.075	2.875 (3 x 0.958)	0.050	3.000	0.5

Raw data table (Excel)

	A_{410nm} at various GPNA concentrations (mM)					
Time (s)	0.5	1.0	1.5	2.0	3.0	4.0
0	0.000	0.000	0.000	0.000	0.000	0.000
30						
60						
90						
120						
150						
180						

Graph 1



- How do we relate A_{410} to [P]?
- Haemoglobin practical
- Beer-Lambert Law

How do we change Absorbance into [P]?

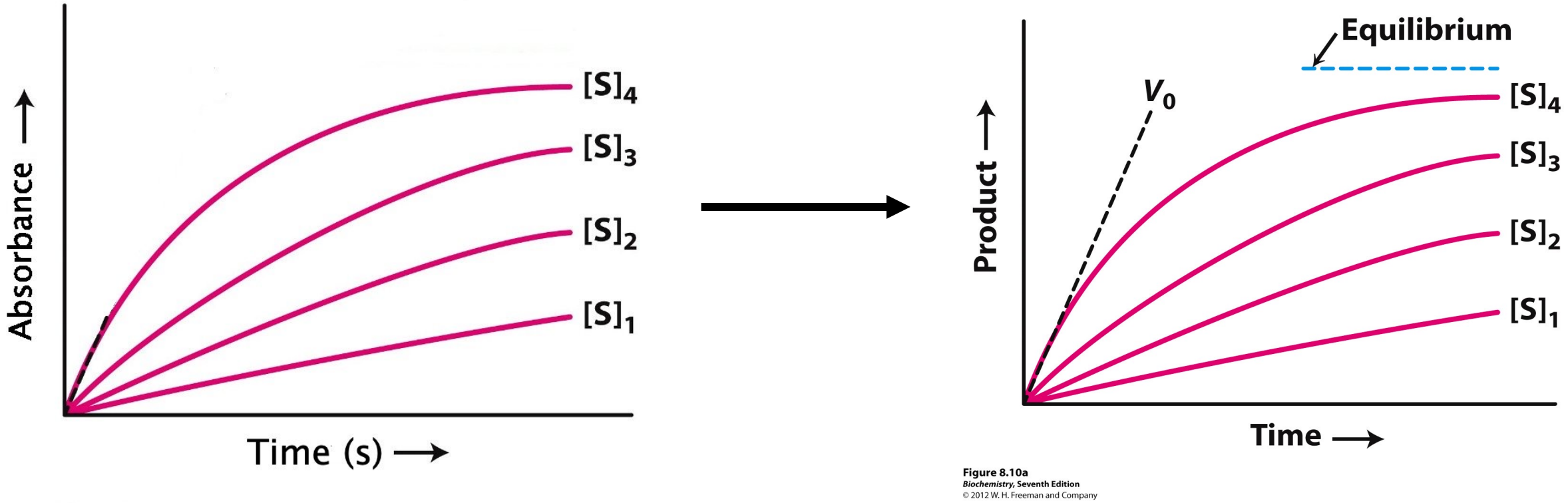
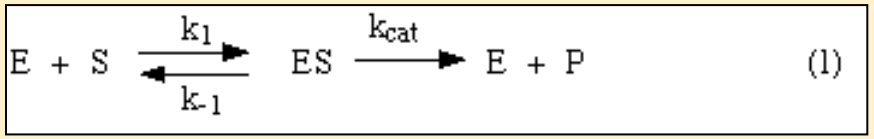


Figure 8.10a
Biochemistry, Seventh Edition

1-POM-5-3: Protein techniques: Explain how the techniques of spectrophotometry and electrophoresis may be used in the study of proteins.

A simple (?) model of enzyme:substrate interaction



For this model, let V_0 be the initial velocity of the reaction. Then
$V_0 = k_{cat}$ [ES]. (2)

The maximum velocity V_{max} occurs when the enzyme is saturated -- that is, when all enzyme molecules are tied up with S, or

$[ES] = [E]_{total}$.
So $V_{max} = k_{cat} [E]_{total}$. (3)

During the initial phase of the reaction, as long as the reaction velocity remains constant, the reaction is in a **steady state**, with ES being formed and consumed at the same rate. During this phase, the rate of formation of [ES] equals its rate of consumption. According to model (1),

Rate of formation of [ES] = $k_1[E][S]$.
Rate of consumption of [ES] = $k_{-1}[ES] + k_{cat}$ [ES].
So in the steady state, $k_{-1}[ES] + k_{cat}$ [ES] = $k_1[E][S]$. (4)

$(k_{-1} + k_{cat})$ [ES] = $k_1[E][S]$,
and $(k_{-1} + k_{cat})/k_1$ = [E][S]/[ES]. (5)

Grouping the kinetic constants by defining them as K_m :

$K_m = (k_{-1} + k_{cat})/k_1$ (6)

and then express [E] in terms of [ES] and [E]total:

$[E] = [E]_{total}$ - [ES] (7)

Substitute (6) and (7) into (5):

$Km = ([E]_{total}$ - [ES]) [S]/[ES] (8)

Solve (8) for [ES]: First multiply both sides by [ES]:

[ES] $K_m = [E]_{total}$ [S] - [ES][S]

Then collect terms containing [ES] on the right:

[ES] K_m + [ES][S] = $[E]_{total}$ [S]

Factor [ES] from the left-hand terms:

[ES](K_m + [S]) = $[E]_{total}$ [S]

and finally, divide both sides by (K_m + [S]):

[ES] = $[E]_{total}$ [S]/(K_m + [S]) (9)

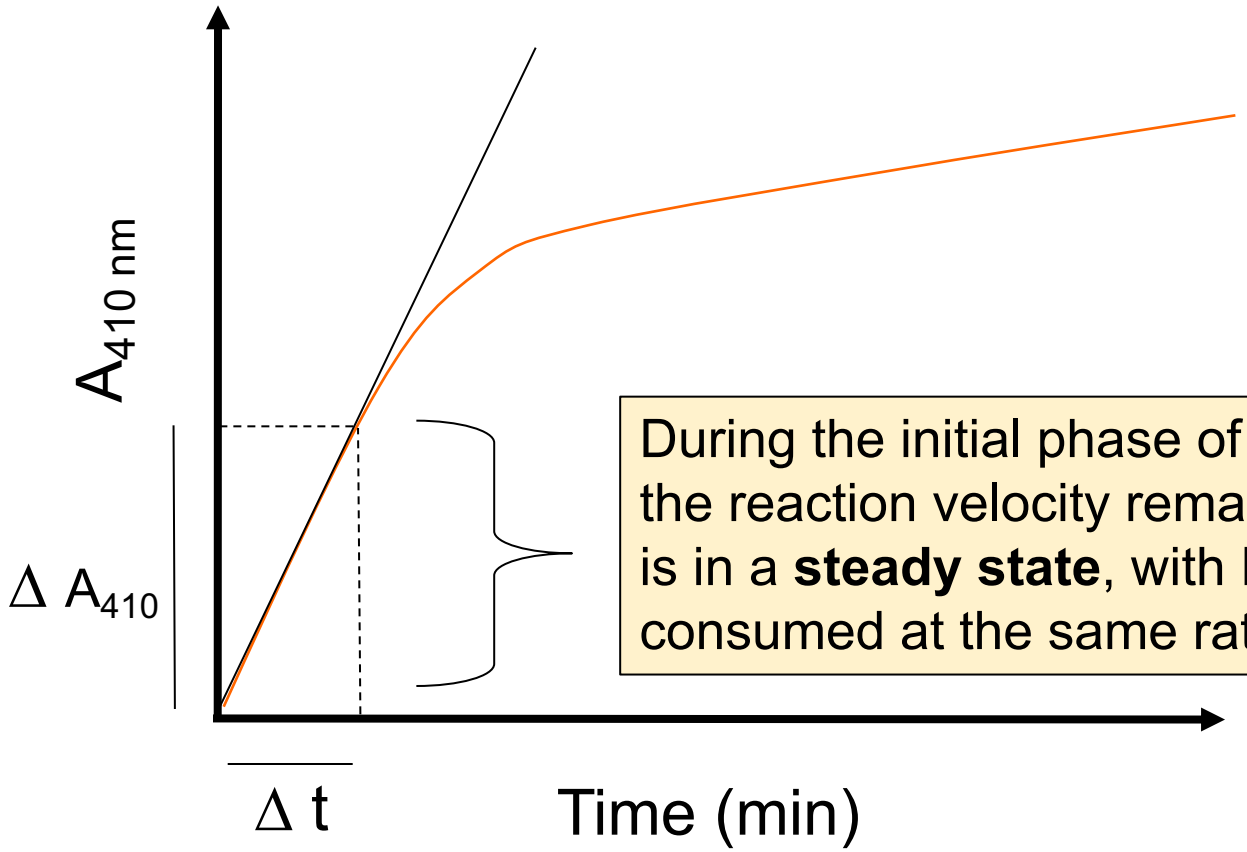
Substitute (9) into (2): $V_0 = k_{cat} [E]_{total}$ [S]/(K_m + [S]) (10)

Recalling (3), substitute V_{max} into (10) for $k_{cat} [E]_{total}$:

$V_0 = V_{max}$ [S]/(K_m + [S]) (11)

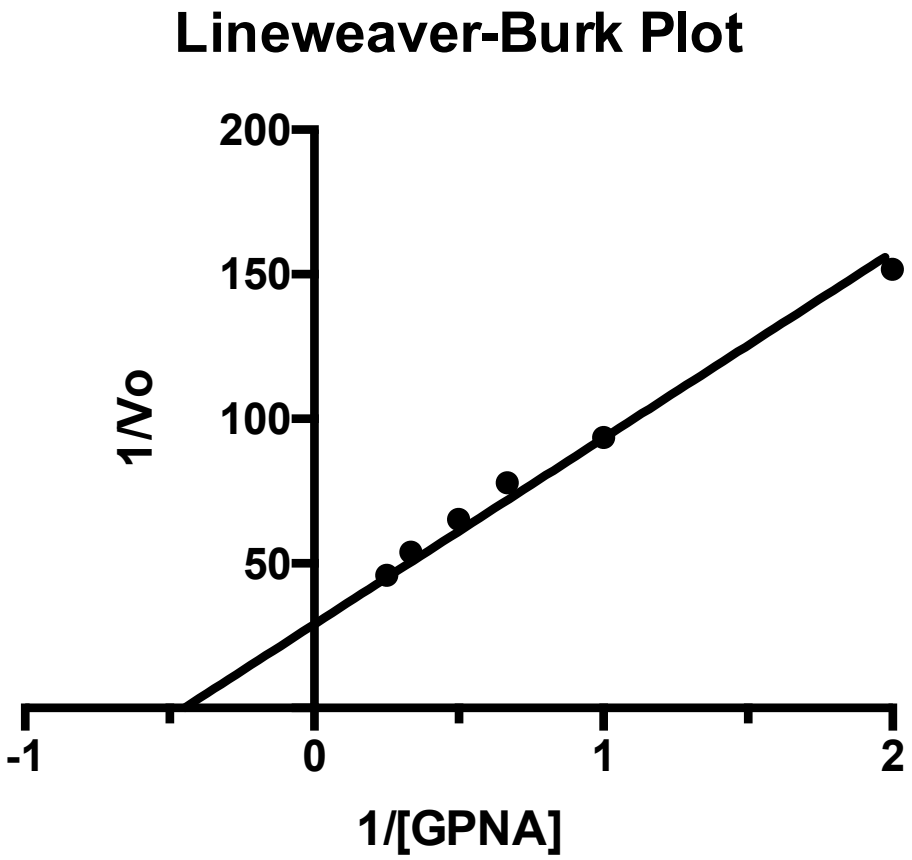
N.B. You do not need to derive or recall this!

Calculating Vo



During the initial phase of the reaction, as long as the reaction velocity remains constant, the reaction is in a **steady state**, with ES being formed and consumed at the same rate.

Graph 2



V_{max} = ?

K_M = ?

Enter your data via the Qualtrics link