

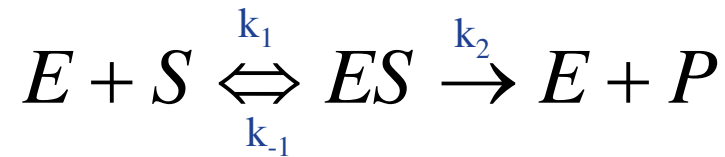
# ITC in Enzyme Characterization



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# Enzyme Kinetics

$K_M$  is analogous to  $K_d$



$$K_M = \frac{k_2 + k_{-1}}{k_1}$$

When  $k_{-1} \gg k_2$

$$K_M = K_d$$



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# Enzyme Kinetics

$V_{\max}$  is proportional to [enzyme]

$$V_{\max} = k_{\text{cat}}[E]$$

$K_m$  is independent of [E]

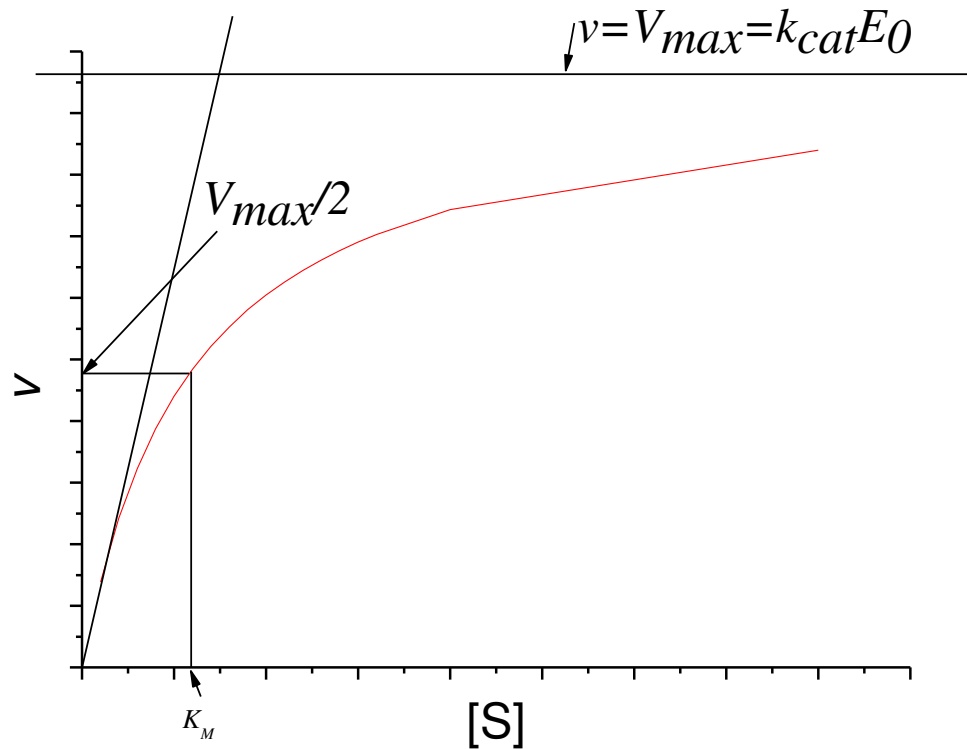
Velocity of reaction is given by Michaelis-Menten equation

$$v = \frac{[S]}{K_M + [S]} k_{\text{cat}}[E]$$



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# Classical Enzyme Kinetics



# Two ITC methods

## Multiple substrate injections

- Low enzyme concentration
- Steady state conditions

## Continuous assay

- Higher enzyme concentration
- Single injection of substrate



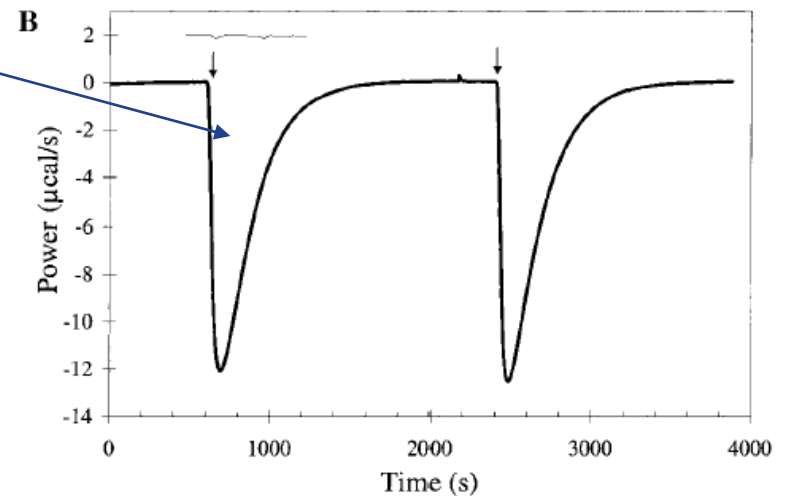
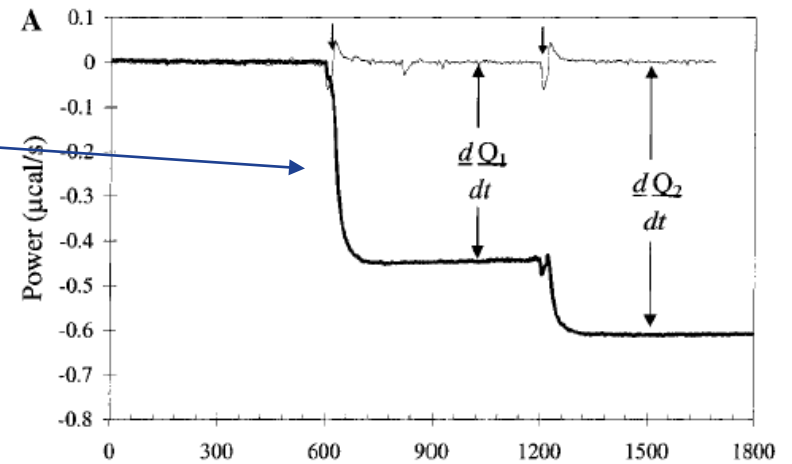
# Enzyme Kinetics By ITC

$$Power = \frac{dQ}{dt}$$

$$Q = n\Delta H_{app} = [P]_{total} V\Delta H_{app}$$

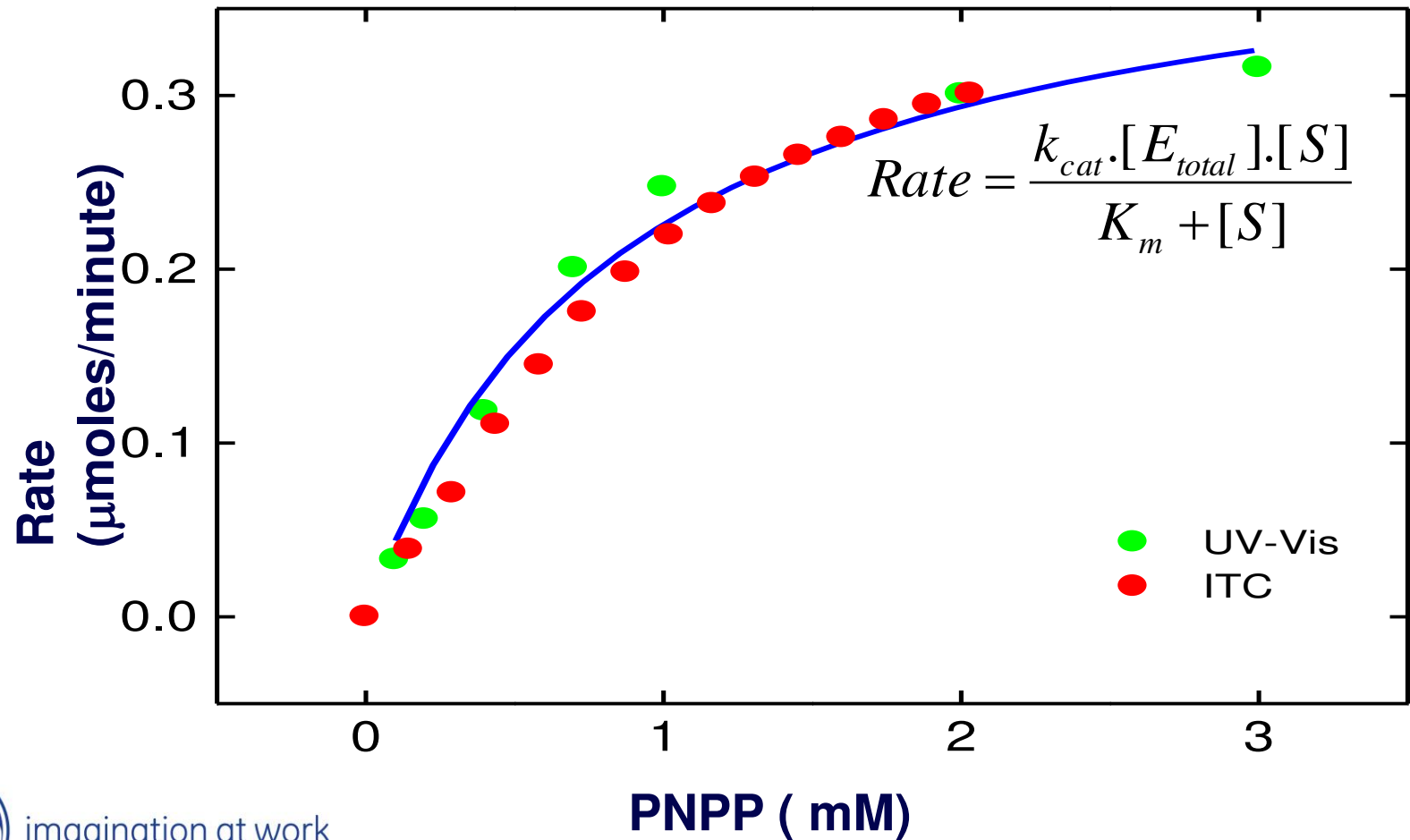
$$\frac{dQ}{dt} = \frac{d[P]}{dt} V\Delta H_{app}$$

$$Rate = \frac{d[P]}{dt} = \frac{1}{V\Delta H_{app}} \frac{dQ}{dt}$$



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# Enzyme Kinetics



# [Enzyme] and [Substrate]

Following each injection of substrate there should be no appreciable depletion of substrate (<5%) prior to the next injection.

However substrate concentration cannot be too high otherwise [S] will be above  $K_m$  after the first few injections.

If enzyme-substrate affinity is high then enzyme concentration needs to be low

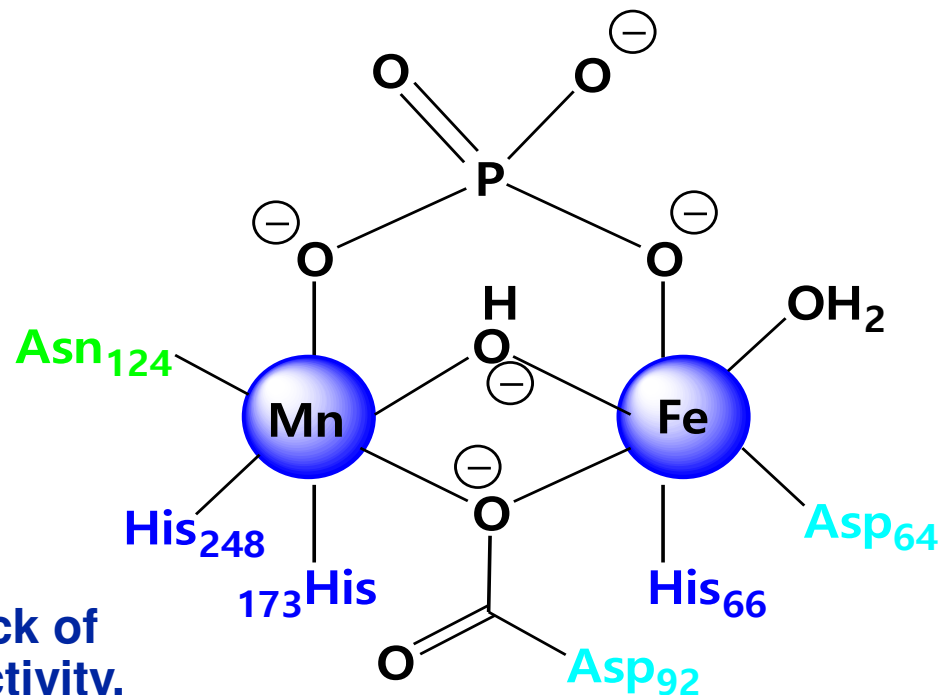
However this relies upon there being a sufficiently large enthalpy to detect.



# PP1- $\gamma$ Phosphatase



Phosphatases catalyse the hydrolytic removal of a phosphate group. In this study we examine a **serine/threonine phosphatase (PP1- $\gamma$ )**.



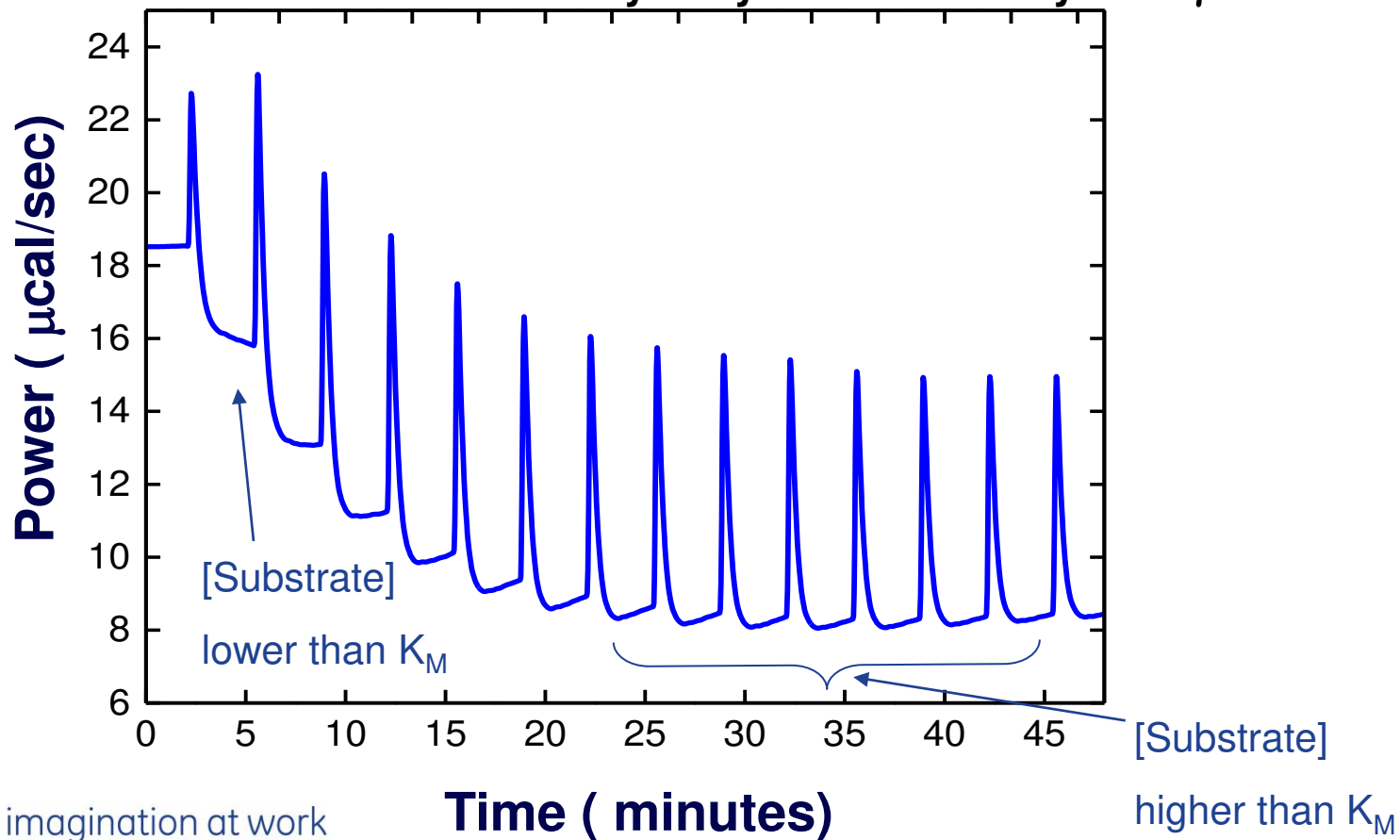
This enzyme catalyses the direct attack of water and it requires metal ions for activity.



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# Enzyme Kinetics

Raw calorimetric data for the measurement of the reaction rate for the hydrolysis of PNPP by PP1- $\gamma$ .



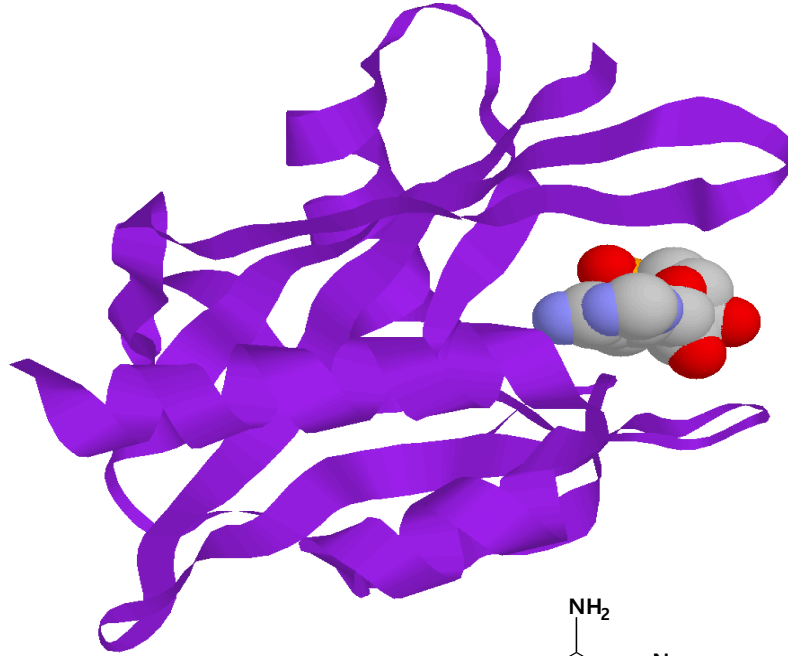
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# Enzyme Kinetics

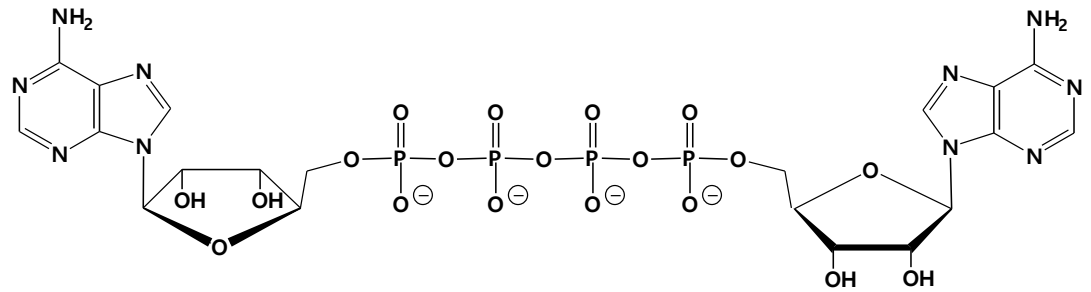
ITC	UV
$K_m = 1.2(\pm 0.2) \text{ mM}$	$K_m = 0.9(\pm 0.2) \text{ mM}$
$k_{\text{cat}} = 0.6 (\pm 0.1) \text{ s}^{-1}$	$k_{\text{cat}} = 0.5 (\pm 0.1) \text{ s}^{-1}$
$V_{\text{max}} = 0.43 \text{ } \mu\text{M min}^{-1}$	$V_{\text{max}} = 0.39 \text{ } \mu\text{M min}^{-1}$



# Enzyme Kinetics

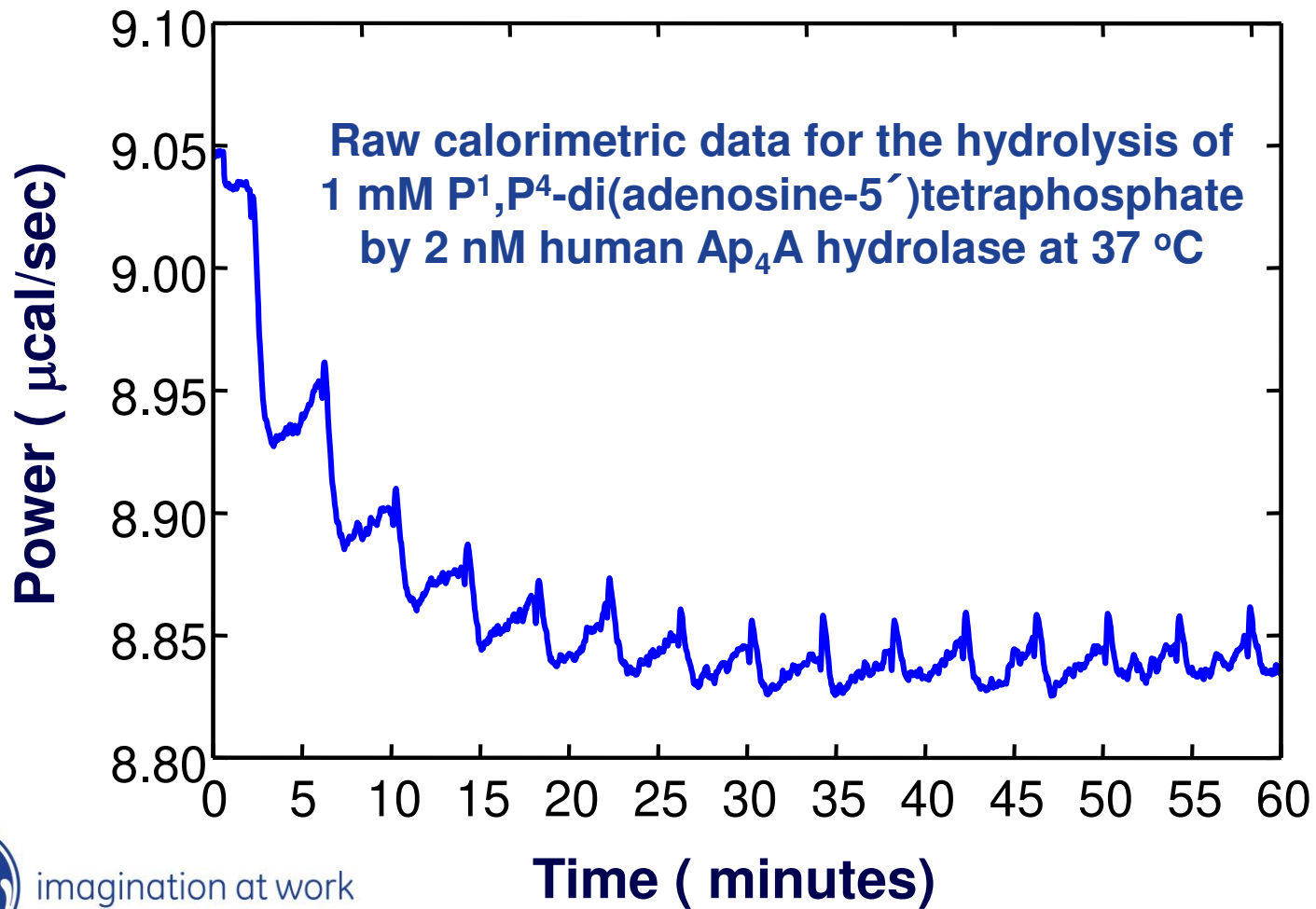


Ap4A Hydrolase

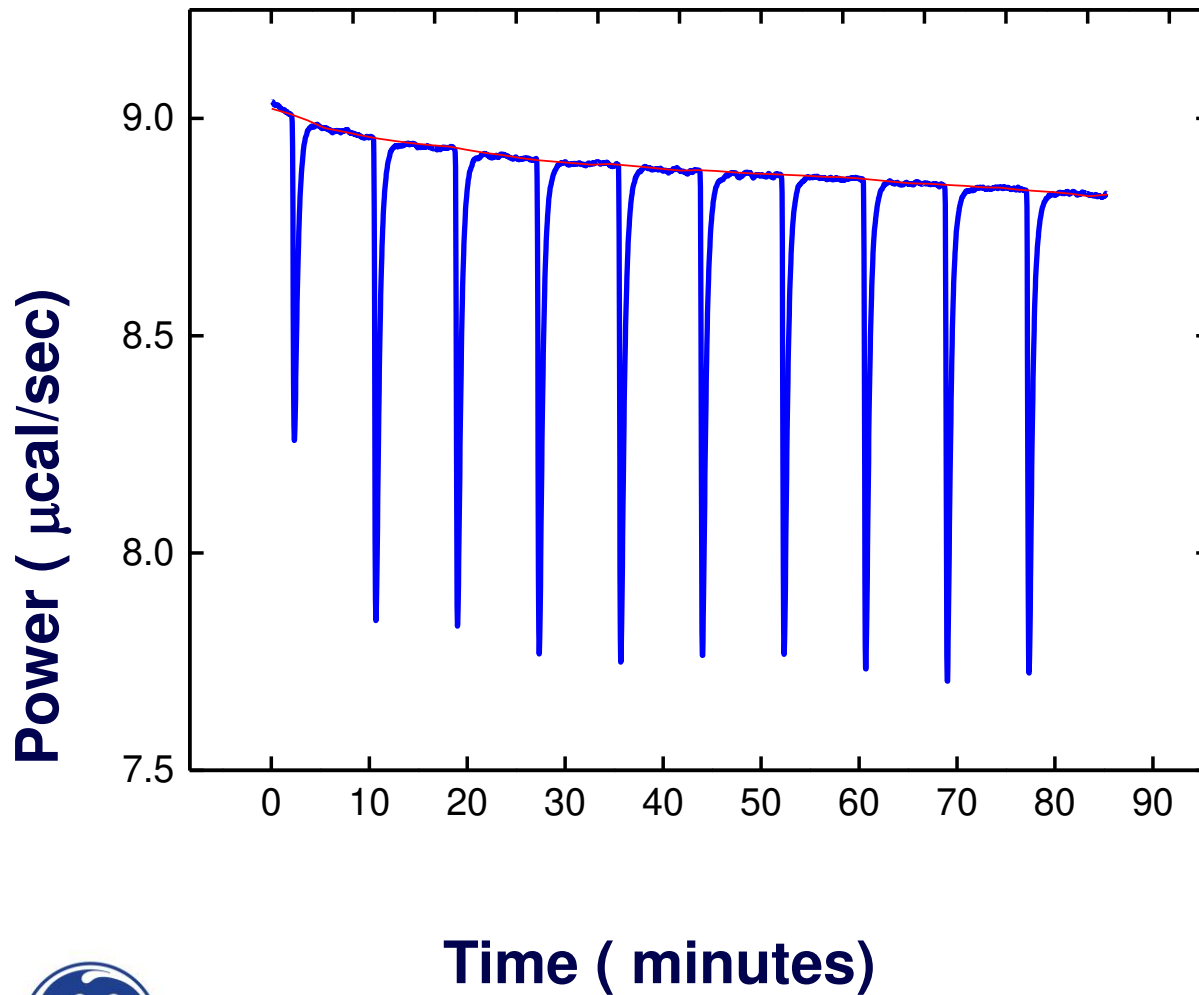


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# Enzyme Kinetics



# Enzyme Kinetics

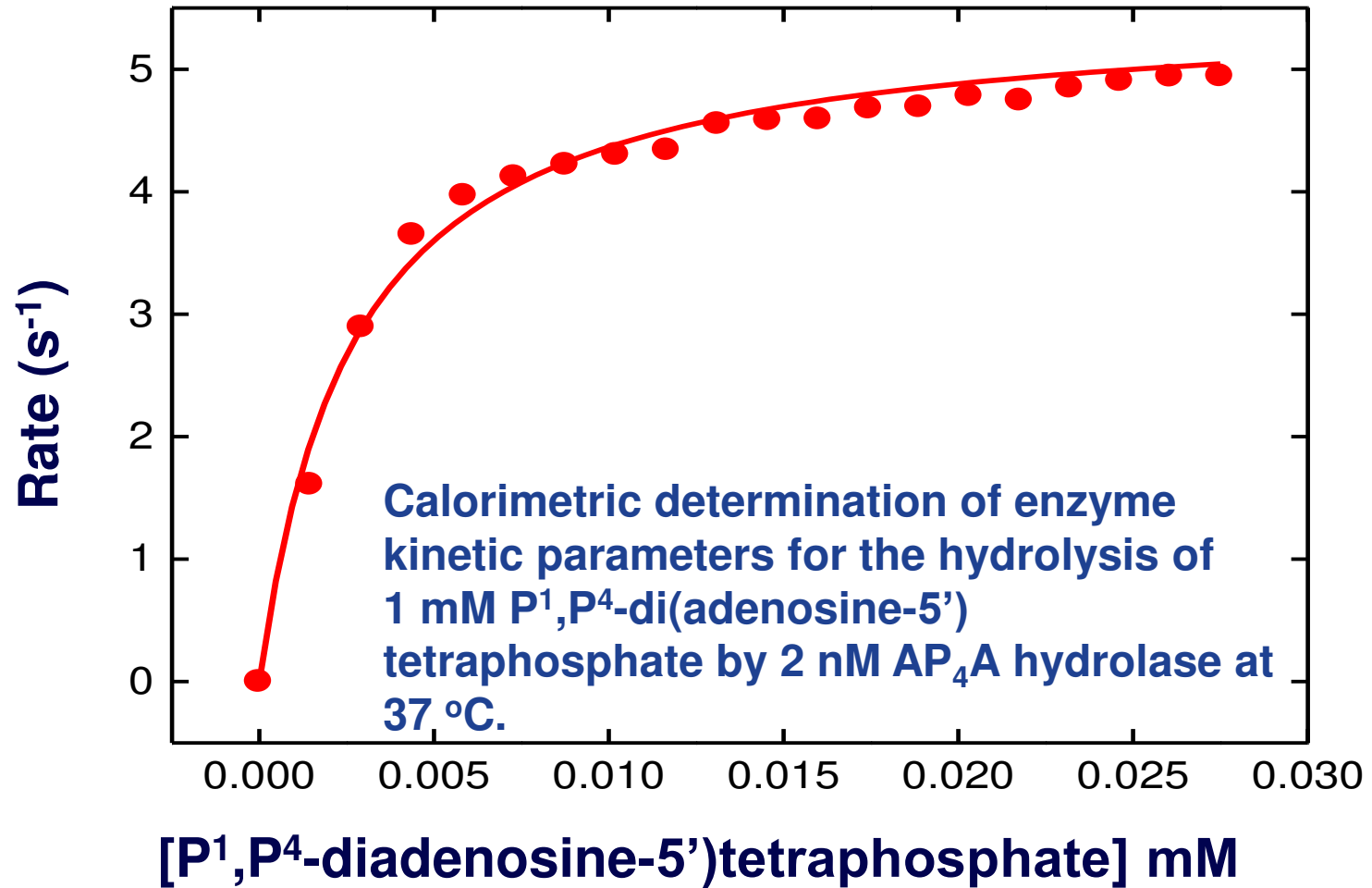


Determination of  $\Delta H_{\text{app}}$   
for the hydrolysis  
of 1 mM P<sub>1</sub>,P<sub>4</sub>-  
di(adenosine-5')  
tetraphosphate  
by 100 nM AP<sub>4</sub>A  
hydrolase at 37 °C

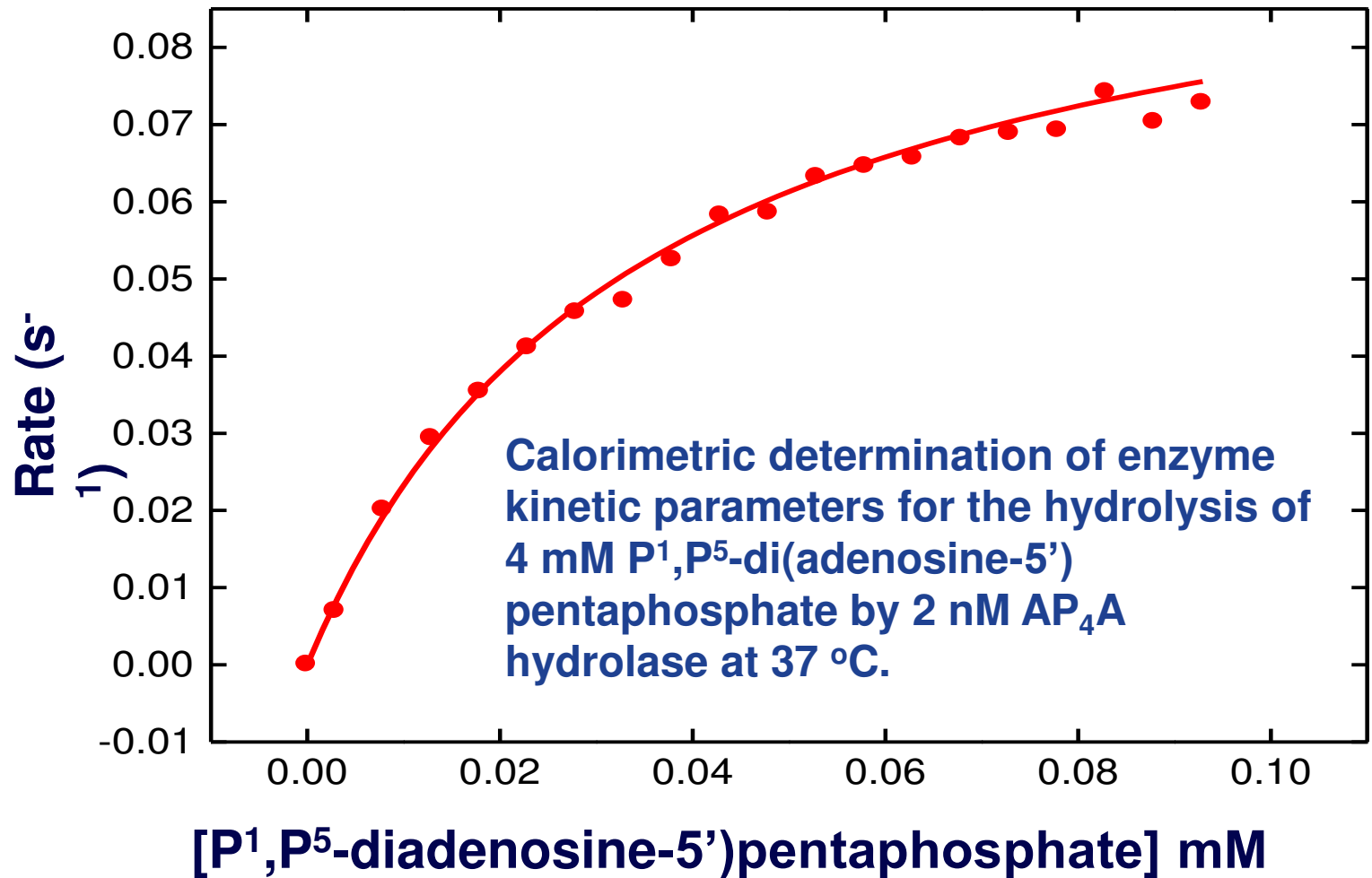


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# Enzyme Kinetics



# Enzyme Kinetics



# Enzyme Kinetics

## Hydrolysis of diadenosine polyphosphates by Ap<sub>4</sub>A Hydrolase

**P<sup>1</sup>,P<sup>4</sup>-di(adenosine-5')**   **P<sup>1</sup>,P<sup>5</sup>-di(adenosine-5')**   **ATP**  
**tetraphosphate**   **pentaphosphate**

$K_m$  ( $\mu\text{M}$ )  
*nd*

$2.68 \pm 0.80$

$34.5 \pm 1.8$

$k_{\text{cat}}$  ( $\text{s}^{-1}$ )  
*nd*

$2.76 \pm 0.03$

$0.05 \pm 0.01$

$V_{\text{max}}$  ( $\text{nM s}^{-1}$ )  
*nd*

5.52

1.0

$\Delta H_{\text{app}}$  ( $\text{kcal mol}^{-1}$ )

$-19.24 \pm 0.27$

$-16.38 \pm 0.74$

-

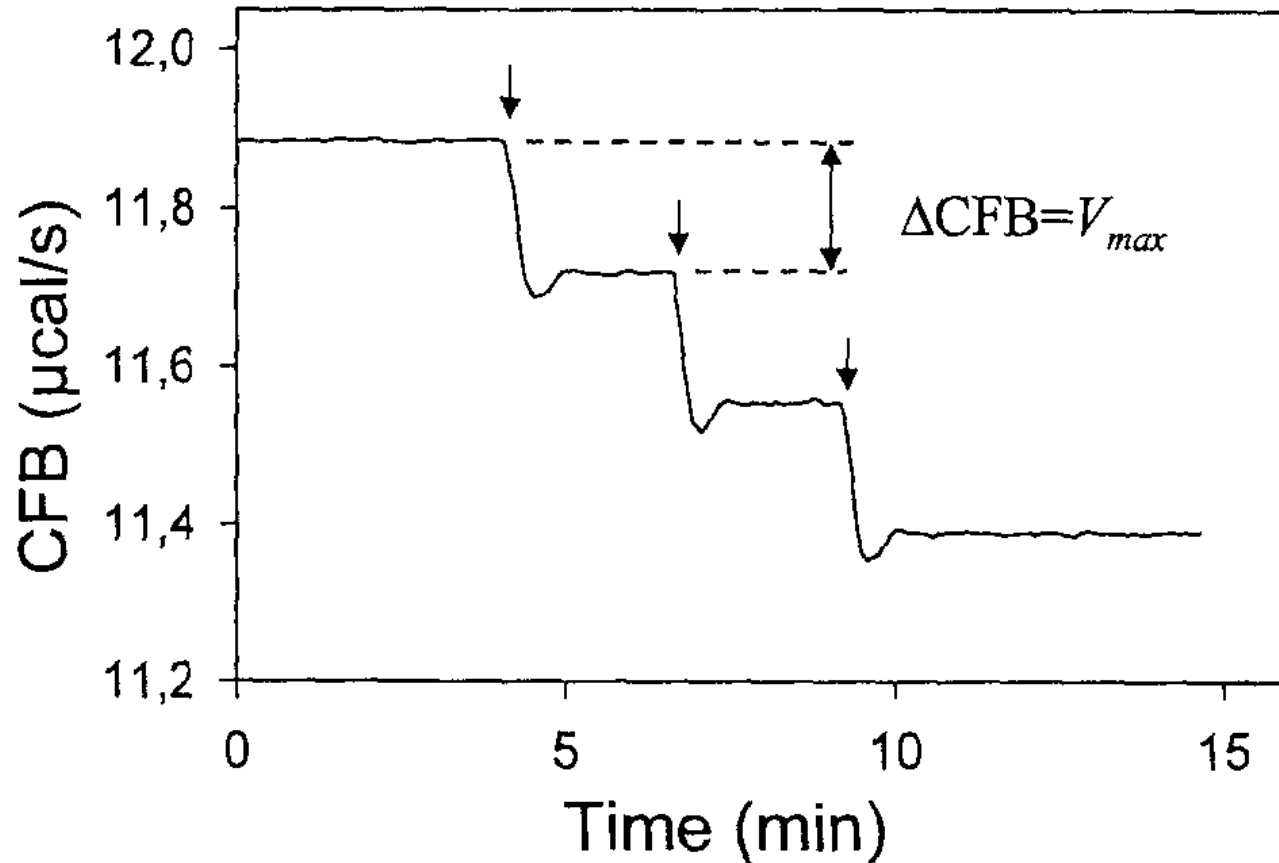
$8.6 \pm 0.2$



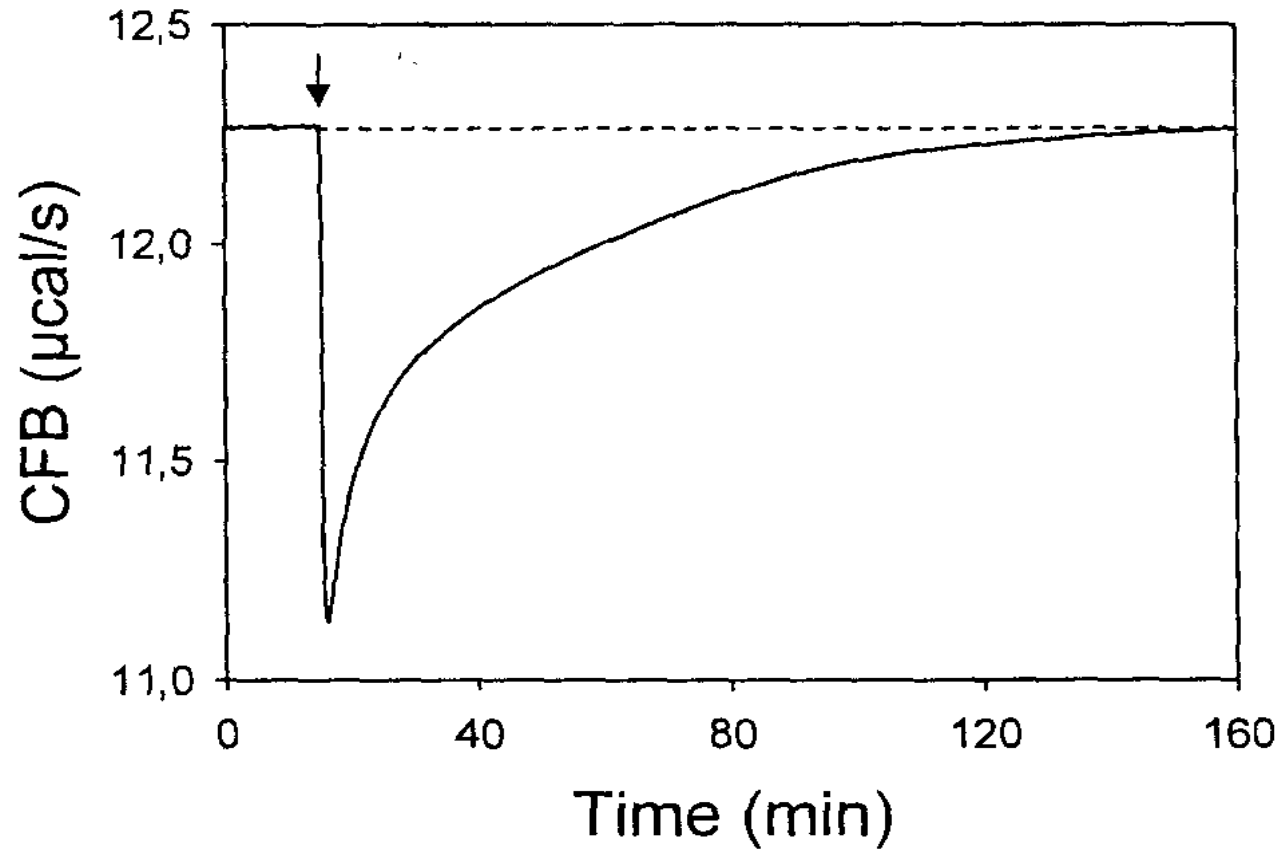
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# Enzyme Kinetics

Alternatively.....

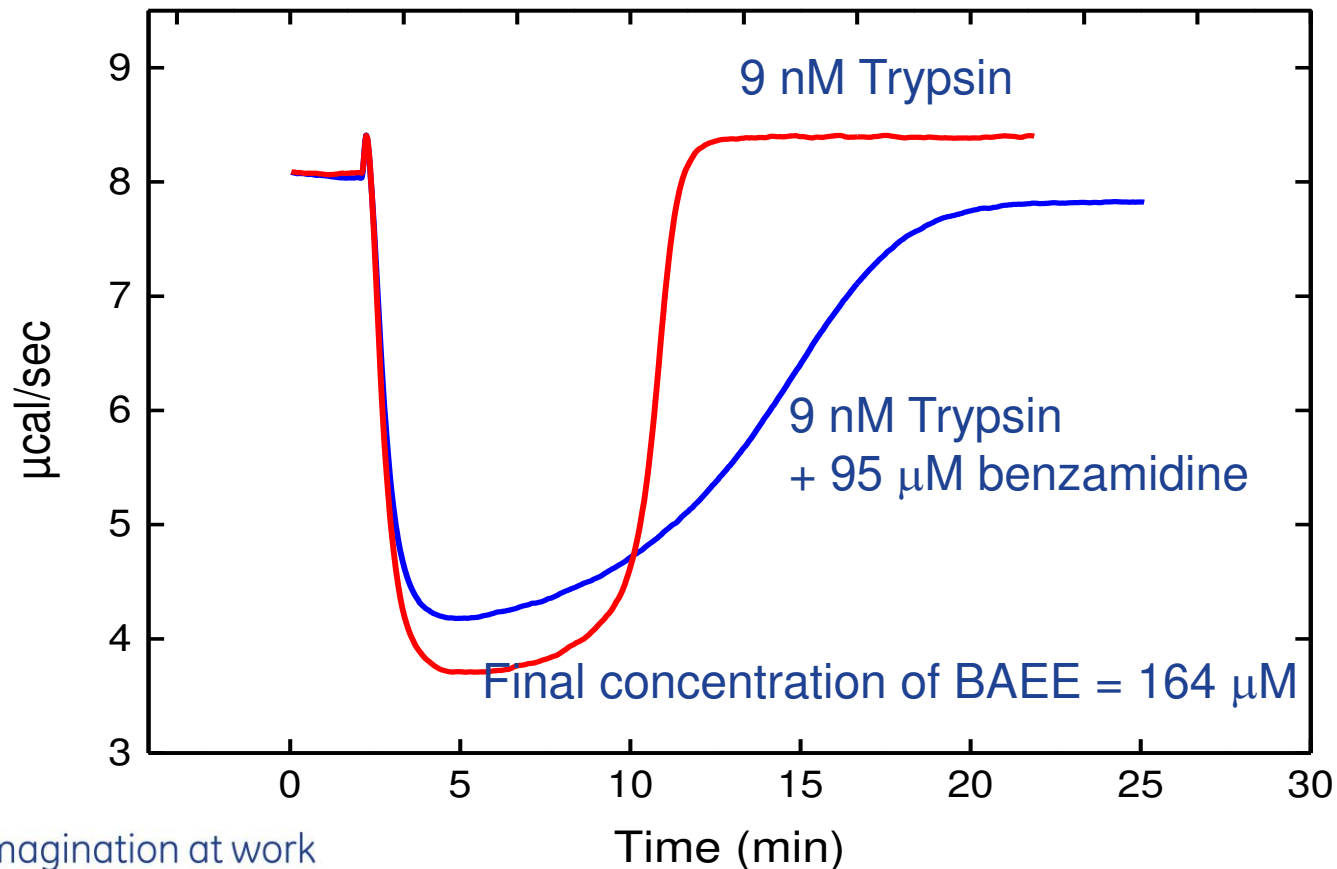


# Enzyme Kinetics



# Continuous Injection Method

Trypsin catalysed hydrolysis of BAEE and inhibition by benzamidine



# Continuous Injection Method

From this raw data the **rate** at any time can be obtained using:

$$Rate = \frac{d[P]}{dt} = \frac{1}{V \cdot \Delta H_{app}} \frac{dQ}{dt}$$

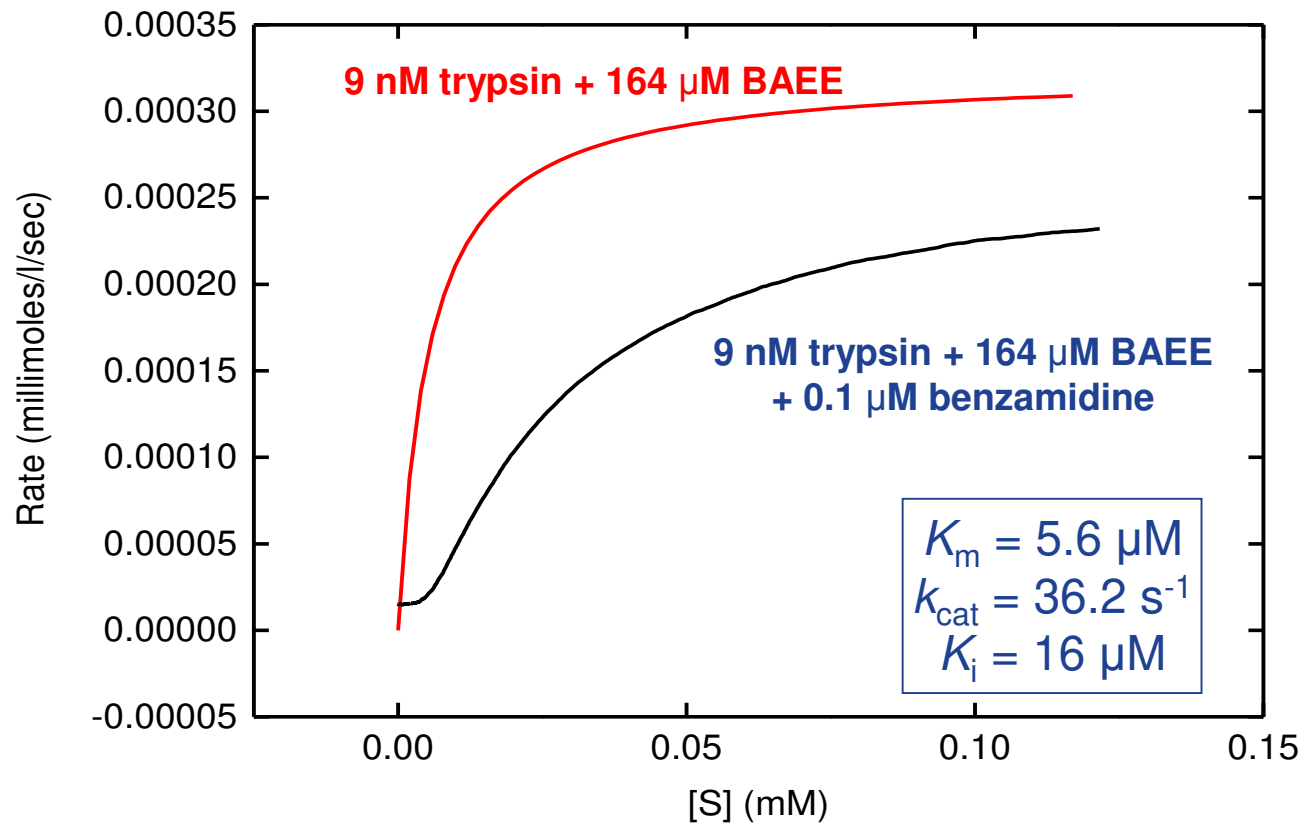
The amount of substrate left at any given time after the initial injection can be determined from the integral of the heat evolved:

$$S_t = S_{Total} - P_{(t)} = S_{Total} - \frac{\int_{t=0}^t Q_{(t)}}{\Delta H \cdot V}$$



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# Continuous Injection Method



# Enzyme kinetics

Universal assay

No need to couple assay to colour change reaction

No labelling required

Modifications to substrate and/or enzyme do not effect assay

Turbid solutions can be used

Pico-femtomoles of protein required

Quick



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# Comparison of calorimetric and other assay data

Enzyme	Calorimetric		Literature Values	
	$K_m$	$V_{max}$	$K_m$	$V_{max}$
E.C. 1.5.1.3 (DHFR) <sup>a</sup> Substrate = DHF	1.2 $\mu\text{M}$	6 $\text{s}^{-1}$	6 $\mu\text{M}$	3 $\text{s}^{-1}$ <a href="#">[i]</a>
E.C. 2.7.1.1 (yeast hexokinase) <sup>b</sup> Substrate = glucose	72 $\mu\text{M}$	270 $\text{s}^{-1}$	100 $\mu\text{M}$	450 $\text{s}^{-1}$ <a href="#">[ii]</a>
E.C.3.3.2.6 <i>B. cereus</i> Penicillinase I <sup>c</sup>	120 $\mu\text{M}$	3600 $\text{s}^{-1}$	50 $\mu\text{M}$	2800 $\text{s}^{-1}$ <a href="#">[iii]</a>
E.C. 3.4.21.4 (Trypsin) <sup>d</sup>	4 $\mu\text{M}$	15 $\text{s}^{-1}$	5 $\mu\text{M}$	22 $\text{s}^{-1}$ <a href="#">[iv]</a>
E.C. 3.4.21.16 (HIV protease) <sup>e</sup> Substrate = KARVnLF(NO <sub>2</sub> )EAnL Substrate = VSQNYPIVQ	5 - 300 $\mu\text{M}$ [NaCl] dependent	10 $\text{s}^{-1}$	15 $\mu\text{M}$	45 $\text{s}^{-1}$ <sup>33</sup>
E.C. 3.5.1.5 ( <i>H. pylori</i> urease) <sup>f</sup>	0.79 mM	1400 $\text{s}^{-1}$	0.17 mM	2700 $\text{s}^{-1}$ <a href="#">[v]</a>
E.C. 4.1.1.7 ( <i>F. heparinum</i> heparinase) <sup>g</sup>	1.8 $\mu\text{M}$	0.059 $\text{s}^{-1}$	10.2 $\mu\text{M}$	92 $\text{s}^{-1}$ <a href="#">[vi]</a>
E.C. 4.1.1.39 (Rubisco) <sup>h</sup> Substrate = ribulose bis phosphate	0.15 mM	1.95 $\text{s}^{-1}$	0.053 mM	1.76 $\text{s}^{-1}$ <a href="#">[vii]</a>
E.C. 4.1.3.18 (Acetolactate synthase) <sup>i</sup>	4.8 mM	11 $\text{s}^{-1}$	5.5 mM	5.3 $\text{s}^{-1}$ <a href="#">[viii]</a>
E.C. 5.99 (GroEL) <sup>j</sup>	3 $\mu\text{M}$ n = 2.9	0.052 $\text{s}^{-1}$	5 $\mu\text{M}$ n = 2.5	0.08 $\text{s}^{-1}$ <sup>22</sup>
E.C. 6.4.1.1 (Pyruvate carboxylase) <sup>k</sup> Substrate = ATP Substrate = pyruvate	85 $\mu\text{M}$ 105 $\mu\text{M}$		58 $\mu\text{M}$ 440 $\mu\text{M}$	<a href="#">[ix]</a>



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Todd and Gomez, Anal. Biochem. 276, 179-187 (2001)