Lecture outline

- **Goal:**
  Demonstrate $K_M$ determination from progress curves using numerical methods.
  Use a "real-world" problem arising from the instructor’s consulting practice.

- **Topics:**
  The Michaelis constant: theory and practice.
  The illusion of linearity in enzyme assays.
  Global vs. local fit of multiple data sets: Mechanistic implications.
  The choice of proper time interval to measure the “initial” rates.
  Assessing the randomness of residual plots.
  Enzyme deactivation mechanisms.

- **Implementation:**
  The DynaFit software package.

The setup: FDA put a “hold” on a drug application

**FDA = UNITED STATES FOOD AND DRUG ADMINISTRATION AGENCY**

We have the following comments, to be addressed prior to submission of a license application:

1. A proposal essay that mentions the kinetic parameters ($K_M$ and $V_{max}$) and uses a graphically oriented advance different and sophisticated manner using an unified program for drug substance and product price to initiation of the Phase 3 clinical trial.

- The drug candidate is an enzyme.
- The company is a small biotech company — no kinetist on staff.

The solution: Hire a consultant to:
- design a biochemical assay; and
- propose a method of data analysis.

The raw data

**The raw data**

* vary 
  + add 
  + dissolve

[Substrate] from 3.5 to 56 µM 
[Enzyme] at 5 nM
5 replicates all 485 rpm

The raw data

$K_M$ ?

Michaelis constant: Mechanistic definition

- **$K_M$ has two different meanings, even for the simplest possible enzyme mechanism:**
  
  \[
  E + S \rightleftharpoons ES \rightarrow E + P
  \]
  
  \[
  K_M = \frac{k_2}{k_1}
  \]

  equilibrium dissociation constant
  
  \[
  K_{cat} = \frac{k_3}{k_1}
  \]

  "strength of enzyme/substrate binding"

  special case: when dissociation ($k_2$) is much faster than chemical reaction ($k_3$)

  \[
  k_2 \gg k_3 \quad \Rightarrow \quad K_M = \frac{k_2}{k_1}
  \]

  $K_M$ .............. substrate binding

  $K_{cat}$ .............. reactivity to form product
Michaelis constant: Experimental definition

IF THE DOSE-RESPONSE CURVE IS HYPERBOLIC, THEN "KM" HAS ONLY ONE MEANING

Michaelis-Menten equation:

\[ v = \frac{k_{cat}[E][S]}{[S] + KM} \]

**The human eye is very bad at seeing nonlinearity**

WE EVOLVED TO SEE STRAIGHT LINES - EVEN WHEN THERE ARE NONE!

If this were a "linear portion", our problems would be over: We could get the initial rate by fitting a straight line to the first 100 seconds.

**Derivatives approximated as “finite differences”**

CHECKING LINEARITY BY SIMPLE COMPUTATIONS

By the time the assay reached 100 seconds, the reaction rate decreased by almost one half!

**The plot of instantaneous rates**

IF THERE WERE A "LINEAR PORTION", THE RATE PLOT SHOULD BE STRICTLY HORIZONTAL

**Trying the straight-line fit anyway**

HOW BAD COULD IT BE, REALLY?

quite bad, actually: This plot should look completely "random"
What about a quadratic polynomial?

**CAREFUL HERE: POLYNOMIALS ARE UNSUITABLE FOR EXTRAPOLATIONS!**

Why would we need to extrapolate?

```
y = -0.00000327x^2 + 0.00192642x + 0.07670882
R^2 = 0.99987003
```

![Graph of a quadratic polynomial](image1)

Mixing delay time

**IN MOST KINETIC EXPERIMENTS WE NEVER “SEE” THE VERY INITIAL TIME POINT**

Enzyme & UV/Vis transparent substrate concentration is the same

Absorbance at time zero should be identical...

... unless a lot of product was formed already at “t = 0”

```
0.07 0.09 0.11 0.13 0.15 0.17 0.19 0.21 0.23 0.25
0 2 04 06 08 10 12 14 16 18
```

![Graph of absorbance over time](image2)

Why would we need to extrapolate?

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```

The devil in the details: Initial reaction rates

**A FEW INNOCENT-SOUNDING CHALLENGES IN ANALYZING THE PROGRESS OF ENZYME ASSAYS:**

1. The time-course of enzyme reaction is nonlinear. It can be considered “almost linear” only in special cases.
2. There is a finite mixing delay requiring extrapolation. In our experiments we always “miss” the initial rate.
3. Extrapolation is dangerous – unless we use a mechanistic model. Generic mathematical models such as polynomials are “out”.

All these problems would disappear if/enough of the progress of enzyme assays can be treated as linear.

This is almost never the case.

Mechanistic mathematical model

**MECHANISTIC MODELS ARE WELL SUITED FOR EXTRAPOLATIONS**

**Michaelis-Menten mechanism:**

\[
\begin{align*}
    & \\
n & \Rightarrow & \text{reaction product} \left( \text{slow} \right) \\ & \Rightarrow & \text{enzyme-substrate complex} \left( \text{slow} \right) \\
& \Rightarrow & \text{enzyme} + \text{substrate} \left( \text{fast} \right) \\
& \Rightarrow & \text{enzyme} + \text{product} \left( \text{fast} \right)
\end{align*}
\]

Mathematical model for reaction progress:

\[
\begin{align*}
    \frac{d[ES]}{dt} &= k_1[E][S] - k_2[ES] \\
    \frac{d[E]}{dt} &= k_3 - k_1[E][S] - k_2[ES] \\
    \frac{d[S]}{dt} &= k_1[E][S] - k_2[ES] \\
    \frac{d[P]}{dt} &= k_2[ES] \\
    \frac{d[T]}{dt} &= -k_3 \frac{[E][S]}{[E][S] + K_M} \\
\end{align*}
\]

Specialized numerical software: DynaFit

**MORE THAN 600 PAPERS PUBLISHED WITH IT (1996 – 2009)**

```
```

Download http://www.biokin.com/dynafit
The Van Slyke – Cullen mechanism

JUST AS GOOD AS MICHAELS-MENTEN

Van Slyke-Cullen mechanism:

\[ E + S \xrightarrow{k_1} ES \xrightarrow{k_3} E + P \]

Mathematical model for reaction progress:

\[ \frac{d[S]}{dt} = -k[E][S] + k[E][E] \]

\[ \frac{d[P]}{dt} = k[E][E] - k[E][S] \]

Application of the Van Slyke-Cullen irreversible mechanism in the analysis of enzymatic progress curves.

DynaFit input:

- task = fit
- data = progress
- [mechanism]

Application of the Van Slyke-Cullen irreversible mechanism in the analysis of enzymatic progress curves.


Global vs. "local" analysis

A VERY IMPORTANT CONCEPT

- Global analysis: analyze all five curves together as a single data set
- Local analysis: analyze each curve individually as five separate data sets

Local analysis to determine initial reaction rates

WE NEED INITIAL RATES FOR THE MICHAELS-MENTEN EQUATION

Reading off the initial rates

"INITIAL RATE" IS COMPUTED AT A SPECIFIC REACTION TIME (E.G. 1 SEC)

Plot of state variables (species concentrations)

ALWAYS EXTREMELY HELPFUL

Logarithmic plot: Identify the steady-state range

WHEN A BIOCHEMICAL ASSAY REACHES THE STEADY-STATE IS SYSTEM-SPECIFIC
Initial rates from local fit of progress curves

FINALLY READY TO COMPUTE THE $K_M$

Fit initial rates – initial estimate

Fit initial rates – results

The Michaelis constant from initial rates

Global fit of all five progress curves

A closer look:

what else we can learn from the same data?
Global fit – residual plot

If you see a residual plot as bad as this one, completely ignore any other result from DynaFit.

Always look at residual plots.

Global fit – model parameters

These "best fit" concentrations are highly improbable:
It has been established that the actual titration error is much smaller than this.

Enzyme deactivation model: Results

These concentration look OK.

Residual plots: A "good" vs. "bad" comparison

with enzyme deactivation
without enzyme deactivation

Examples of characteristics shown by unacceptable residuals behavior:


"Good" and "bad" residual plots

ASSESSING RESIDUAL PLOTS IS ONE OF THE MOST IMPORTANT SKILLS OF A DATA ANALYST

GOOD: random sequence no "trend" or "pattern"

BAD: non-random sequence shows a "trend" or a "pattern"
Concentration plot: How much deactivation?

Example assay: [E] = 1.4 µM

denatured enzyme is the main molecular form at the end of the assay

What about \( k_{\text{cat}} \)?

**THE FDA WANTS US TO DO TWO THINGS:** \( K_M \) AND \( k_{\text{cat}} \)

**Michaels-Menten equation:**

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

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

The problem:

Even with the differential-equation model, we can (at best) measure only \( V_{\text{max}} \) and \( K_M \).

\( k_{\text{cat}} = \frac{V_{\text{max}}}{[E]} \)

The solution:

At some point we must determine the concentration of active enzyme \([E]\) (active site concentration).

The only other possibility:

1. Determine “apparent \( k_{\text{cat}} \)” from \([E]\) at total protein, for a reference sample of enzyme.
2. Relate every other “apparent \( k_{\text{cat}} \)” value to the reference sample in the future.

\( K_M \) determination: Summary

1. The progress curves must be treated as nonlinear.
   There is no “linear” portion, even if it seems that there is one.
2. Michaelis constant, \( K_M \), was determined by two independent methods:
   - A local fit of progress curves — fit of initial rates (\( K_M = 13.1 \) µM)
   - A global fit of progress curves (\( K_M = 13.6 \) µM)
   Both methods gave the same results.
3. Global fit revealed that the enzyme undergoes deactivation.
   Deactivation (denaturation) is almost complete after 15 minutes.
4. Deactivation does not interfere with \( K_M \) determination.
   If we use the initial rate method; or
   If we take deactivation into account in the global fit.
5. Overall: This substrate is very suitable for a \( K_M \) assay.
   If we use an appropriate (nonlinear) method of data analysis.

Summary and conclusions

1. Even a “simple task” such as \( K_M \) determination can be quite tricky if it is to be done correctly and rigorously.
2. DynaFit can help in dealing with the nonlinearity of reaction progress.
3. Local fit of individual progress curves is not sufficiently powerful to reveal the true kinetic mechanism.
4. Global fit of multiple curve is much preferred for mechanistic studies.
5. Residual plots are crucially important for model validation.
6. DynaFit is not a “silver bullet”: You must still use your brain a lot.