Experimental Methods in Kinetics (2014)

Initial examples assume start $R = A_0 + B_0$ etc., $P = 0$
follow reaction forward → initial rate, etc. measure $[A]$ vs. t

Chemical methods: Could mix and take aliquots,
to gain time quench reaction (T, pH, some change. . )
then use chemical analysis
  Problem slow, requires a lot of material

Bio applications, chemical methods not work so well,
  -- often the change studied not chemically very different
  (folding, enzymology, ligand or membrane binding, . . )
  -- Amounts often very limited (problem for chemical anal.)

Physical Methods: Monitor Absorbance, fluorescence, pH,
  pressure, electrochemistry, scattering, …
  whatever is proportional to concentration
  e.g. Beer-Lambert law: $A = \varepsilon bc$, $A \sim c$
pH, pressure, conduct. are ~ conc., low conc. $I_{Fluor} \sim c$

Mixing limitations:  
Mix reagents in a beaker and stir  
OK for slow reaction  

Slow reaction → pour together and stir  
takes time ⇒ $k_2$ small (seconds)

Speed up by reducing size,  
depend on diffusion - separation
**Fast reaction need - mix fast**

Faster—mix very small volume

*rapid mixing*—small mixer, fast flow & turbulence

Stopped flow (Atkins Fig 21.1-21.2)

– fill cell quickly from mixer
– then stop (cell full of fresh mixed reagents) ⇒ \( t = 0 \)

*Monitor* absorbance, fluorescence or other properties

Something proportional concentration

**Stopped-Flow methods**

—idea is *rapid mixing*,

fill/monitor *small volume*

*Drive* two components (or more) into *mixing chamber* and out to a cell for monitoring a physical property—

typically *absorbance* or *fluorescence*

Mixing can occur in about 1 ms, key is turbulence
Example: Protein folding – rapid mix

Lipid vesicle + β-lactoglobulin, sheet - helix change
(Ning Ge, Xiuqi Zhang, TAK, Biochemistry, 2010)

Circular Dichroism fit to single exponential  \[ [A] = [A_0]e^{-kt} \] – decay in seconds - apparent 1st order – lipid~constant

<table>
<thead>
<tr>
<th>Rate Constant ( k_1 ) (s(^{-1}))</th>
<th>Amplitude (Milideg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.15 mM</td>
<td>0.253±0.011</td>
</tr>
<tr>
<td>0.25 mM</td>
<td>0.159±0.055</td>
</tr>
<tr>
<td>0.5 mM</td>
<td>0.206±0.007</td>
</tr>
<tr>
<td>1 mM</td>
<td>0.194±0.002</td>
</tr>
<tr>
<td>2 mM</td>
<td>0.140±0.001</td>
</tr>
</tbody>
</table>

Fluorescence senses two steps in mechanism, fit to double exponential  \[ [A] = [A_0]\{a'e^{-kt} + a''e^{-k''t}\} \]

\[ N_\beta \rightarrow I_\beta \rightarrow I_\alpha \rightarrow U_\alpha \]  
fast decay: 100 ms, slower one: seconds – like CD

Complex process - use this to postulate a mechanism
Continuous flow – measure along flow $x \rightarrow t$

positions in flow $\Rightarrow$ successively later in reaction, $x \rightarrow t$

Faster
—continuous flow,
more material

Convert

distance to time

$x \rightarrow t$

possible to get to
100μs or better with
micro fluidics

Time scales ~1 ms mixing for small volume: ~ 0.1 ml

Faster $\Rightarrow$ micro mixers $\Rightarrow$

$\rightarrow$ micro etch channels – flow

idea – rate limit mix – diffuse A,B together

$-$ D $-$ different constant $\sim$ constant

$-$ shorter distance between A,B (mixing)

Same Concept – follow conc. [A] vs. t - physical probe
determine rate, vary [A] / determine order
Example: rapid mix β-lactoglobulin+TFE, β→α transition

Continuous flow mixer

Top view:
green: inlet channels,
red: 8μm deep outlet channel

Side view: 2D Fluid dynamics simulation

%TFE
Austin, Gerwert
PNAS 98
2001, 6646

Lifetimes of intermediates in the β-sheet to α-helix transition of β-lactoglobulin using diffusional IR mixer

E. Kauffmann, N. C. Darnton, R. H. Austin, C. Batts, and K. Gerwert

PNAS 2001 98 6646-6649

a) Spectra along channel: 1.1, 3.4, 5.7, 10.2, 21.6, 103 ms
b) 2nd deriv. & 3-state fit
c) 3 basic spectra derived
d) Time course of 3 states
Speed up initiation—if photochemical initiate 
A → A* then this can be created in very short time

Bio-exam: Hb-CO → Hb* + CO → recombine after photolysis

Rhodopsin → Rh* --photo absorb → cis-trans conform. change

Photocycle – light pulse trigger, measure rates for spectral changes, e.g. retinal absorbance, can also trap intermediates with cold or other environment

Above focus on loss of reagent (or forming product)
If reverse reaction or alternate steps important
Need to monitor other species (intermediates)

Equilibrium - rate depend on forward and reverse step: R & P
Equilibrium, if elementary

A + B \xrightarrow{k_1/k_{-1}} C + D \quad r_{\text{for}} = r_{\text{rev}} \quad \frac{r_f = k_1 [A][B]}{r_r = k_{-1} [C][D]} = 1

\[ K_e = \frac{k_1}{k_{-1}} = \frac{[C]e[D]e}{[A]e[B]e} \]

Disturb equilibrium – perturbation – then \textit{relax back to equil.}

- change T, P, pH, ... how? \rightarrow \text{discharge capacitor (T), shock wave (P), Laser flash-(pH precursor) or ex. T-jump (laser absorb by solvent)}
- system \textit{relax to new equilibrium}
- reaction must go forward/reverse to new state (new equil. conc.)

Unimolecular case:

A \xrightarrow{k_1/k_{-1}} B \quad A = A_e - x \quad B = B_e + x

x = departure from new equilibrium at time = t, new T, P

\[ x = x_e e^{-t/\tau} \quad \tau \text{- relaxation time, } x \rightarrow 0 \text{ at } t = \infty, \quad x = x_0/e \text{ at } t = \tau \]

\[ r = k_1A - k_{-1}B = k_1(A_e-x) - k_{-1}(B_e+x) \]

\[-dA/dt = dx/dt = -(k_1+k_{-1})x + k_1A_e - k_{-1}B_e = -(k_1+k_{-1})x \]

\textit{Since at equil. } k_1A_e = k_{-1}B_e, \textit{ can drop 2nd and 3rd terms}

\[ dx/x = -(k_1+k_{-1})dt \rightarrow \ln[x/x_0] = -(k_1+k_{-1})t \rightarrow \]

\[ x = x_0 e^{-t(k_1+k_{-1})} \rightarrow \text{relaxation time: } 1/\tau = k_1 + k_{-1} \]

Note: relax faster if either \( k_1 \) or \( k_{-1} \) fast (same as rate to equil.)
(see \textit{Derivation 21.4}, p.797 in Atkins, Table 7.4 in Tinoco or Engel Ch. 25.11) – 2\textsuperscript{nd} order more complex form
\textit{(hint: } x\text{-small, so } x^2 \text{–very small, neglect quadratic, } 2\textsuperscript{nd} \text{order})
Since $K_{eq} = k_1/k_{-1}$ get both values $k_1$, $k_{-1}$ from $\tau$ & $K_{eq}$

Slow T-jump example:
Refold Ribonuclease A, FTIR
Monitor change 2 frequencies

Inverse T-jump: Refolding initiated by injecting
Ribo A stored in syringe at 80 °C into IR cell at 25 °C

One single beam spectrum (IF scan) is collected for each time point.

Time resolution ~ 50 ms, IR resolution separate coil
decay sheet folds

Alternate Example—
see Engel p.687 – RNA conformation

Faster—Dyer, Callender and co-workers protein
and peptide folding with IR or fluorescence

See slides – Webpage
Peptide folding example
UIC T-jump example: TrpZip2 hairpin with isotope label
Labels let us focus on parts of hairpin fold

Variation in Temperature  \( \rightarrow \) Arrhenius behavior

Dynamics Labeled TZ2C
\(^{13}\text{C}=\text{O} \) dynamics picks out character of region
Blue—\( \beta \)-strand
Red—disordered
Green—\(^{13}\text{C}=\text{O} \) relaxation
Helix-Coil mechanism

If a polypeptide is helical, then each residue has local \((\phi, \psi)\) torsions that fit the helical model. If it undergoes a transition to a new form, such as a coil, then the local \((\phi, \psi)\) values change. These could occur in a concerted (all together) manner:

\[
\ldots \text{hhhhhh.} \ldots \rightarrow \ldots \text{cccccc.} \ldots
\]

Or more likely in a stepwise pattern:

\[
\ldots \text{hhhhhh.} \ldots \rightarrow \ldots \text{hchhhh.} \ldots \rightarrow \ldots \text{hcchhh.} \ldots \text{etc.} \ldots \text{cccccc.} \ldots
\]

Residues on the termini are more likely to shift from helix to coil due to solvent exposure and lack of H-bond. Shows up as activation energy:

\[
E_a(\text{hhh.} \rightarrow \text{chh.}) < E_a(\text{hhh.} \rightarrow \text{hch.})
\]
\[
E_a(\text{ccc.} \rightarrow \text{chc.}) < E_a(\text{ccc.} \rightarrow \text{hcc.})
\]

So helices are likely to unfold from the termini, or are more likely to fold from center out to ends.

start unfolded, random coil

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Fig. 5. The conformations at steps 6m, 7m, 8m, 9m, 10m and 40m, from left to right, during the simulation without the 10–12 hydrogen bonding potential. The initial conformation is fully extended. The N-terminus is at the bottom.
or start fully extended:

key is initiate with H-bond formation, center favored:

Taken from: Constant Temperature Simulations of Helix Folding