Lifetimes of free radicals & excited states in biology:

when fast means slow and glowing means snuggling up

Peter Wardman

Our text for today

Conclusions

THINK about
Thermodynamics and Kinetics
For Understanding and Success

(From: Oxygen 2001 *Sunrise Free Radical School*

Free Radical Basics: Concepts and Considerations

Garry R. Buettner, Ph.D.)

http://www.medicine.uiowa.edu/FRRB/SRFRS/SRFRS-papers/SFRS-2001-BuettnerG.pdf

Kinetics: quantitation of radical reactions

- **What happens?**
  - ruling in/out likely/unlikely reactions
- **How fast?**
  - rate constants, concentrations, timescales
- **How much?**
  - competing and reversible reactions, rate-limiting steps, steady-state concentrations
- **How far?**
  - lifetimes and diffusion distances
- **How near – and where?**
  - mapping oxygen, protein/protein interactions

An acronym-free zone – but a few symbols

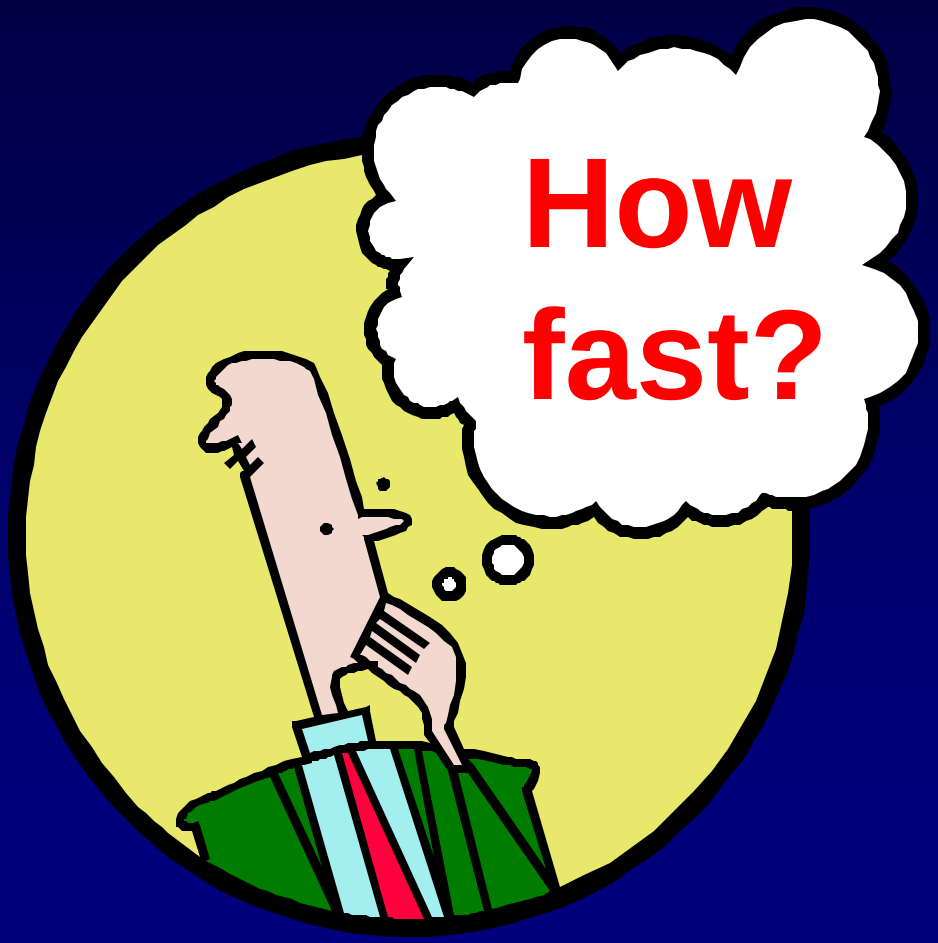
- **Common symbols**

k	rate constant (n.b. lower case)
K	equilibrium constant (upper case)
[X]	concentration of species X
$t_{½}$	half-life
?	lifetime of excited state = 1 / k

- Linking **rate constants** to reaction numbers

k_n	rate constant of reaction (n)
k_{-n}	rate constant of reverse reaction of equilibrium (n)
k_f (k_r)	rate constant of forward (reverse) reaction of equilibrium

Rate constants, concentrations, timescales



Rate constants (coefficients) are the key

- The **rate** of a reaction is often proportional to concentration (denoted by square brackets)
  - A ? product(s)
    - rate of loss of A ? [A]
      = k [A]
  - A + A ? product(s)
    - rate of loss of A ? $[A]^2$
      = $2k$ $[A]^2$ (n.b. *k or 2k ?*)
  - A + B ? product(s)
    - rate of loss of A (B) ? [A] [B]
      = k [A] [B]
- The **rate constant** k quantifies this proportionality
  - the larger the value of k, the higher the reactivity

Rate is not the same as rate constant

- **Rate** of a reaction is the rate of formation of a product, or rate of loss of a reactant
  - units: concentration (molar if in solution) per unit time
- **Rate constant** (*k*, not *K*) characterizes reactivity rather than the rate under specific conditions
  - units vary with reaction type
    - s^{-1} for unimolecular decay
    - $M^{-1}\ s^{-1}$ ($dm^3\ mol^{-1}\ s^{-1}$) for bimolecular reactions
- Even (especially?) experts often wrongly use '**rate**' when they should use '**rate constant**'
  - reactions with high rate constants are not always fast

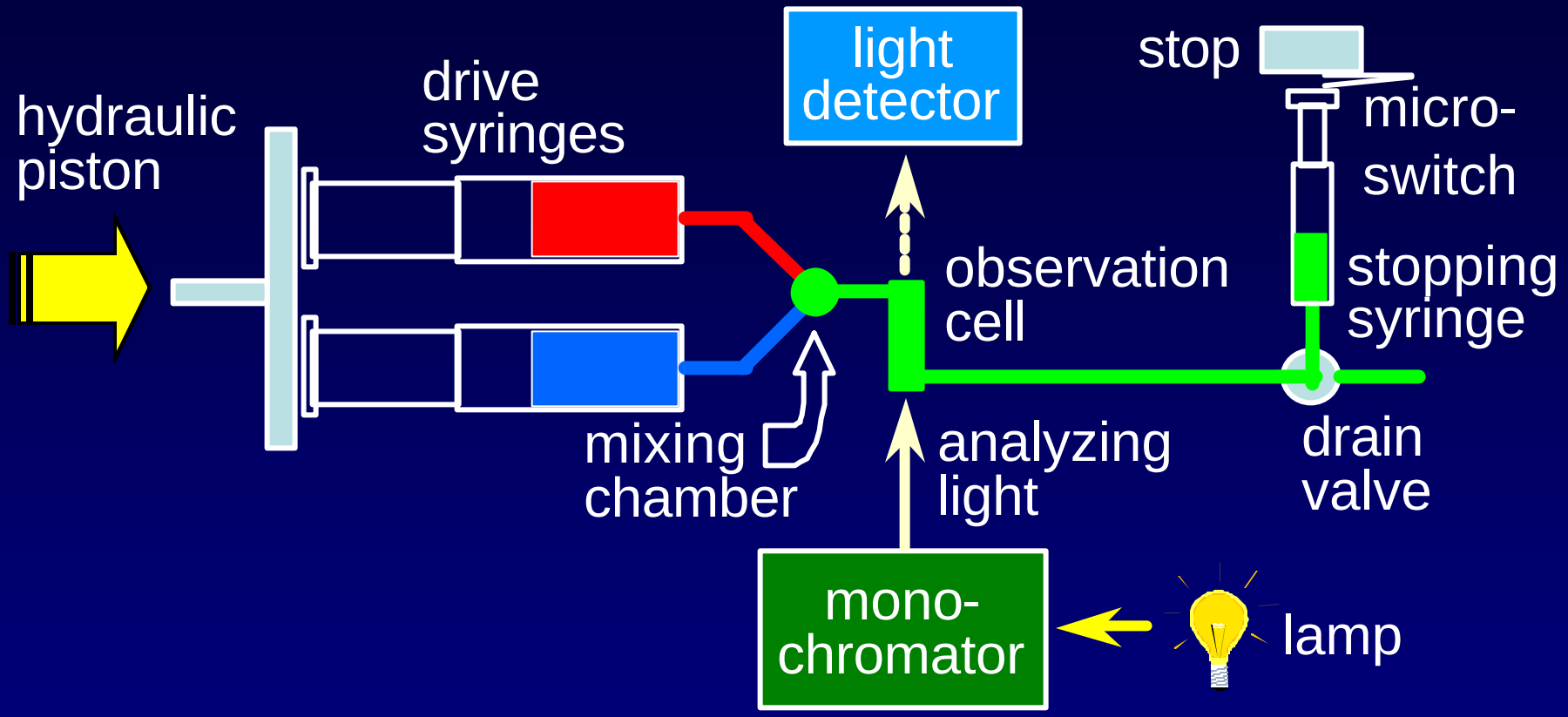
Rate constants: pounds before pennies

- Rate constants can span many **orders of magnitude**, so the ***exponent*** is most important:
  - $k(O_2^{\bullet -}$ + ascorbate) = 5·0 ? 10 **4** M^{-1} s^{-1}
  - $k(O_2^{\bullet -}$ + nitric oxide) ? (3·8 to 15) ? 10 **9** M^{-1} s^{-1}
- **Upper limit:** reactions limited only by diffusion of species (related to viscosity ?, k_{diff} ? 8 **R***T* / (3 ? ?))
  - k_{diff} for reaction in water ~ 7 ? 10^9 to 3 ? 10^{10} M^{-1} s^{-1}
- Most experimental values at **room temperature**
  - *k* for electron transfer from nitroarene radical anion to oxygen increases ~ 2-fold between 25°C and 37°C

Rate constants of radical/fast reactions

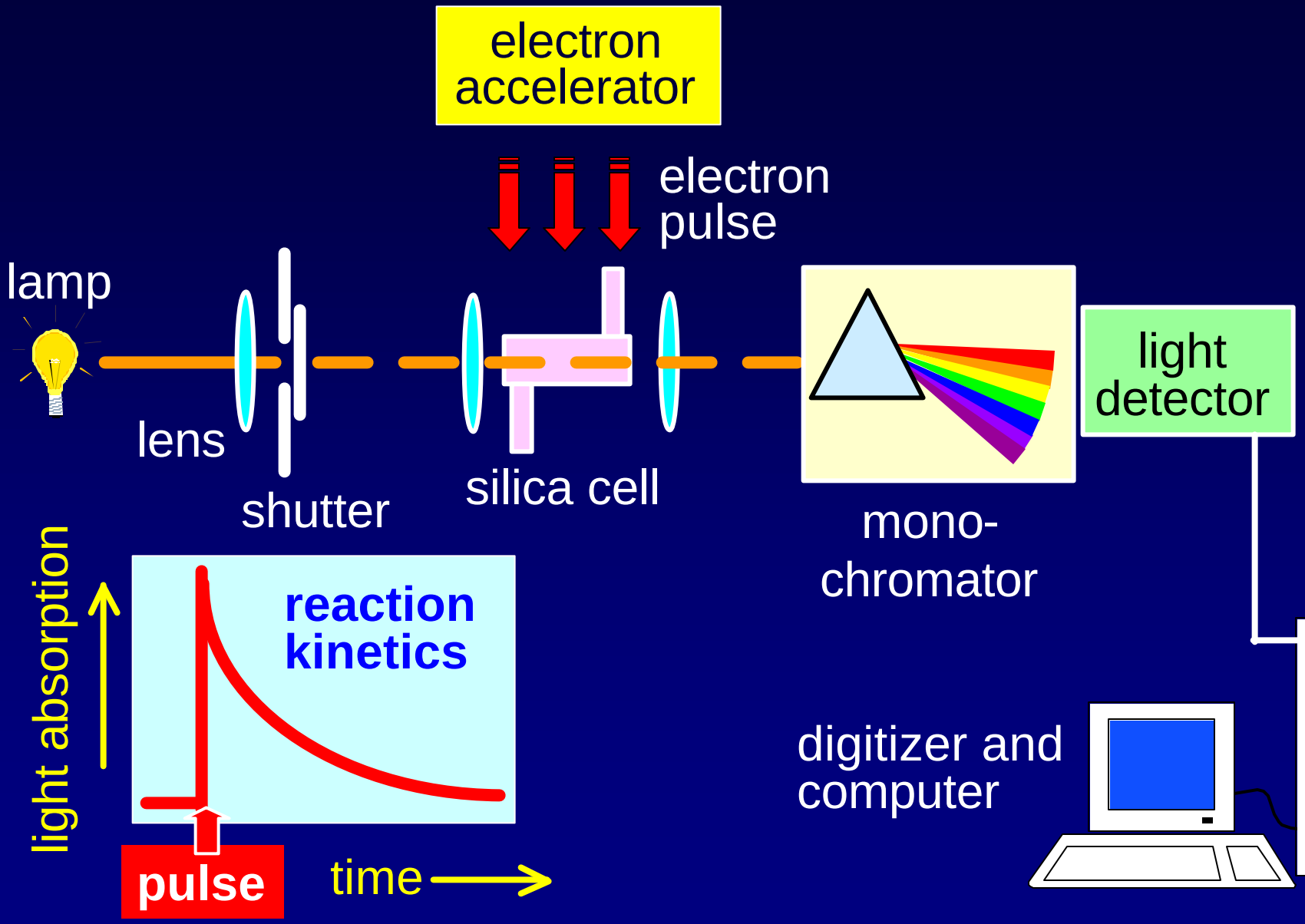
- Monitor **radical, reactant or product *vs.* time**
  - most radicals are short-lived, or reaction is fast
  - generate radicals in short time (pulse, flash)
  - needs high time resolution (micro- to milli-seconds)
- Measure **stable product** during/at end of reaction
  - two competing reactions (known reference)
- Measure **concentrations at steady-state**
  - needs information about competing reactions
  - steady-state concentrations may be extremely low

Stopped-flow rapid-mixing



- Time resolution limited to about 1 ms by interval between mixing and observation

Pulse radiolysis

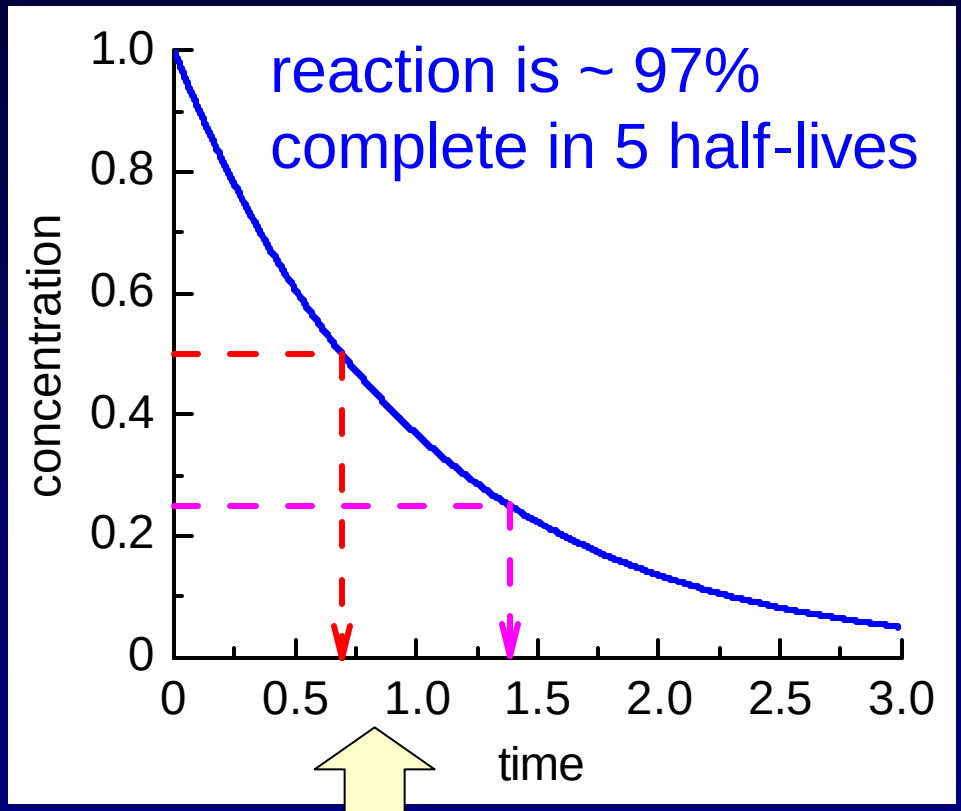


Compilations of rate constants (solution)

- University of Notre Dame
  Radiation Chemistry Data Center
  - web databases from compilations published in the
    Journal of Physical and Chemical Reference Data
  - http://www.rcdc.nd.edu/browse_compil.html
  - http://kinetics.nist.gov/solution/index.php
- Becoming dated and not very user-friendly

Many radical reactions are exponential

- A ? product(s)
  - $t_{½}$ = half-life
    = (ln 2) / k
    ? 0·7 / k
- A + B ? product(s)
  - Radical concentration much less than that of target?
  - If [B] >> [A]
    $t_{½}$? 0·7 / (k [B])



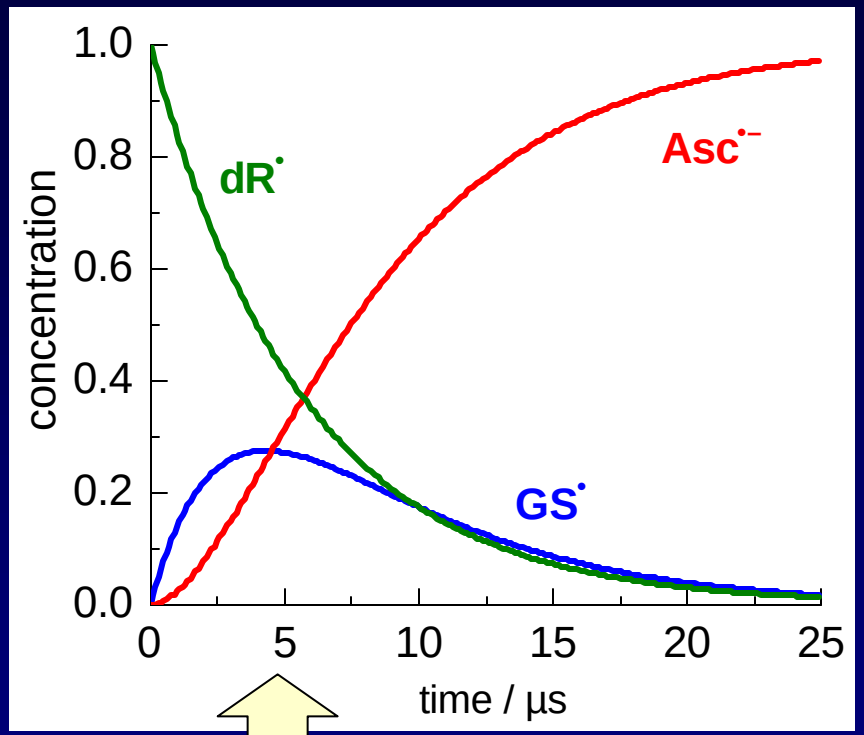
Half-life does not change with concentration of A

Examples of radical lifetimes

- **$^{\bullet}$OH + deoxyribose ? dR$^{\bullet}$**
  k ? 2·5 ? 10^9 M^{-1} s^{-1}
  If [deoxyribose] = 0.1 M
  $t_{½}$? 3 ns (? 0·7/(k [dR]))

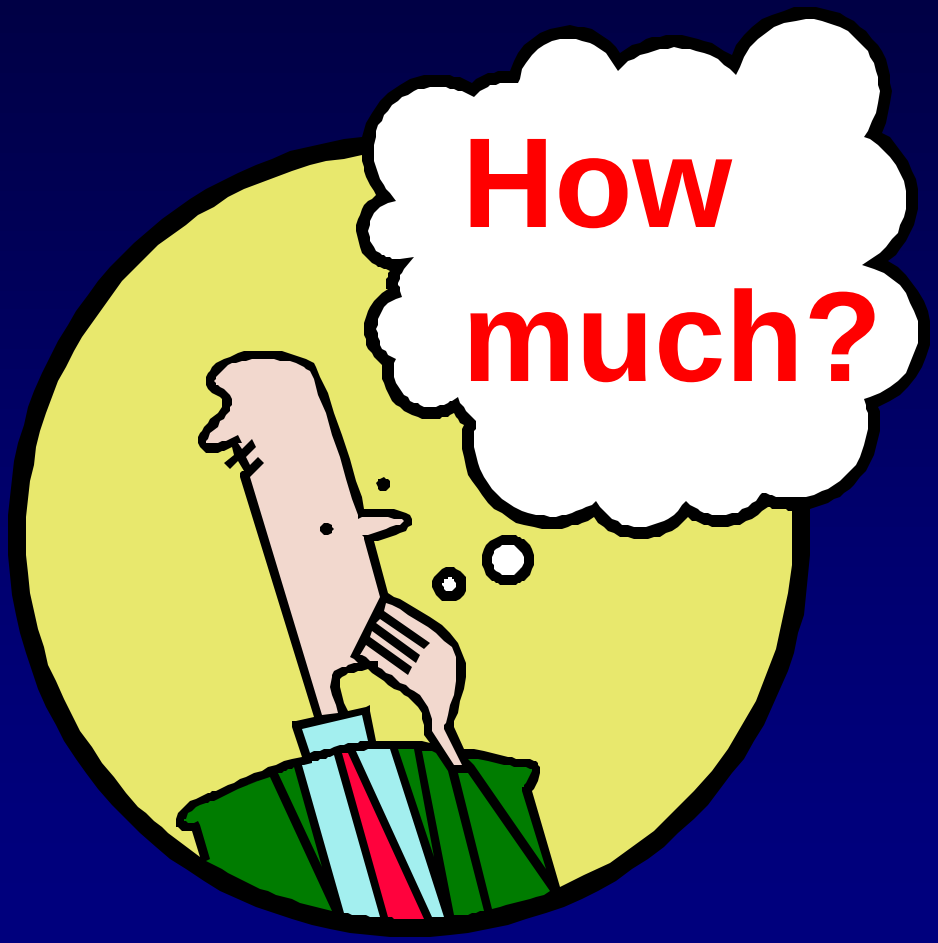
- **dR$^{\bullet}$ + GSH ? dRH + GS$^{\bullet}$**
  k ? 3·5 ? 10^7 M^{-1} s^{-1}
  If [GSH] = 5 mM
  $t_{½}$? 4 µs

- **GS$^{\bullet}$ + AscH^{-} ? GSH + Asc$^{\bullet-}$**
  k ? 6·0 ? 10^8 M^{-1} s^{-1}
  If [AscH^{-}] = 0.5 mM
  $t_{½}$? 2 µs



Intermediates in reaction cascade may have very low concentrations

Competing and reversible reactions, rate-limiting steps, steady-state concentrations



Competition kinetics: *relative* rate constants

- Two competing reactions:

$R^{\bullet} + A \rightarrow$	measurable product, P	k_1
$R^{\bullet} + B \rightarrow$	another product	k_2

- Measure yield of P at any time:

$$[P] \propto \frac{\text{rate of reaction of } R^{\bullet} \text{ producing P}}{\text{sum of rates of all competing reactions of } R^{\bullet}}$$

$$[P] = [P]_0 \frac{k_1 [R^{\bullet}] [A]}{k_1 [R^{\bullet}] [A] + k_2 [R^{\bullet}] [B]}$$

$[P]_0$ = yield in absence of B

$$\frac{[P]_0}{[P]} = 1 + \frac{k_2 [B]}{k_1 [A]}$$

Plot $[P]_0$ / [P] *vs.* [B] / [A]
slope = rate constant *ratio* k_2 / k_1

Rate-limiting steps

- Many reactions involve multiple steps
  - overall reaction rate may reflect the slowest or *rate-determining* step
- Example: **reaction of $NO^{\bullet}$ with GSH**
  - complex reaction forming GSSG and N_2O
  - reaction *may* involve:

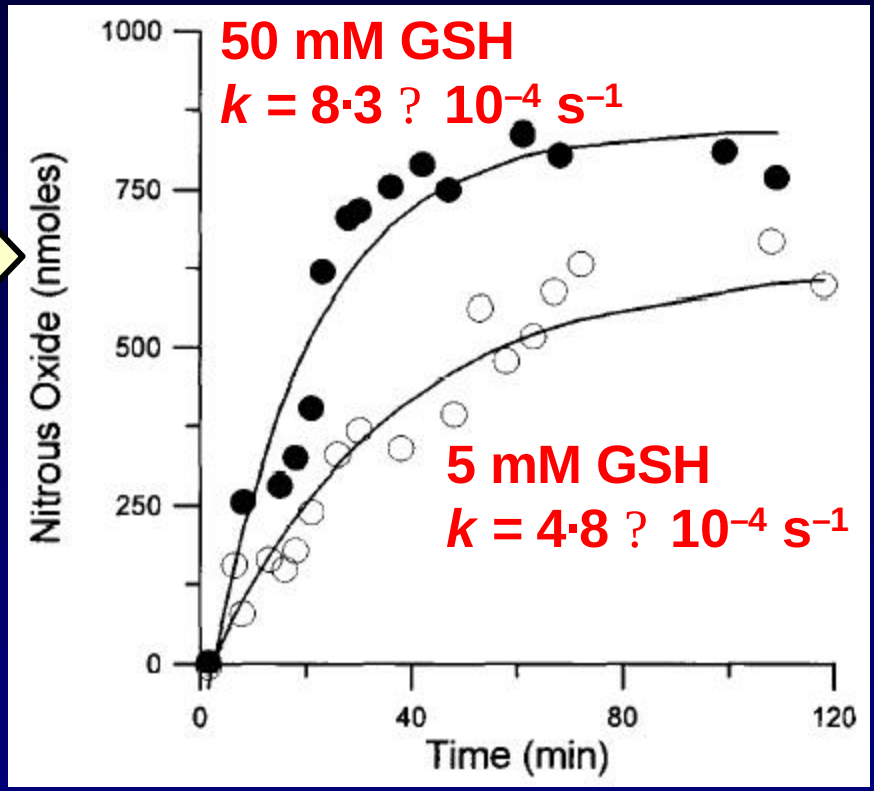
    $GS^- + NO^{\bullet}$ (+H^+) ? GSN$^{\bullet}$OH

    2 GSN$^{\bullet}$OH ? GSN(OH)-N(OH)SG ? GSSG + $H_2N_2O_2$

    $H_2N_2O_2$ (hyponitrite) ? $N_2O + H_2O$
  - may obtain apparently different kinetics depending on whether loss of $NO^{\bullet}$, loss of GSH, or formation of N_2O is measured, and on the concentrations of reactants

Reaction of $NO^{\bullet}$ with GSH: N_2O formation

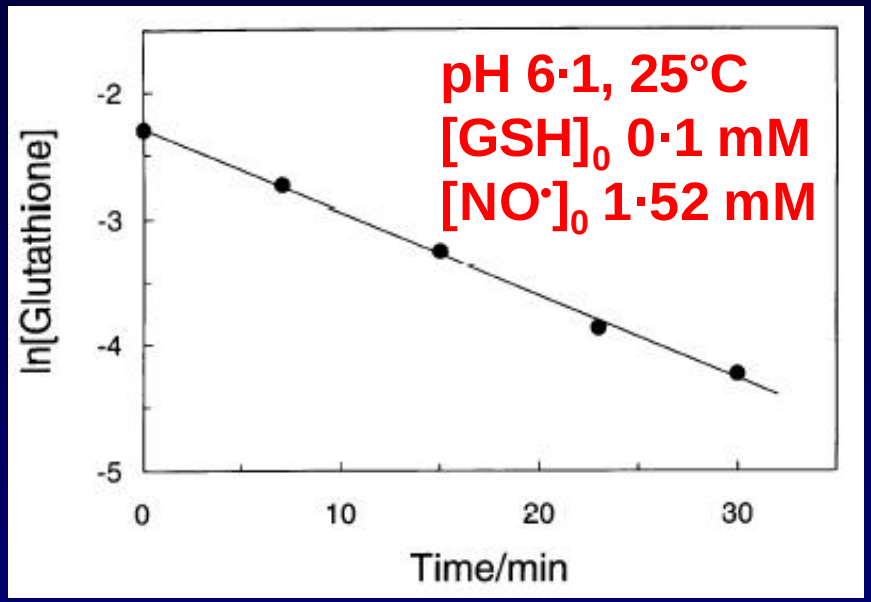
- Hogg *et al.** measured N_2O, with [GSH] >> [$NO^{\bullet}$]: but rate not proportional to [GSH] at high [GSH]
- Possible explanation: hyponitrite decomposition becoming rate-limiting
- Hughes and Stedman† measured pH- and temperature-dependence for: $H_2N_2O_2$? $N_2O + H_2O$ k ? 2–3 ? 10^{-3} s^{-1} at pH 7·4, 37°C



* *FEBS Lett.*, **382**, 223 (1996)
† *J. Chem. Soc.* 129 (1963)

Reaction of $NO^{\bullet}$ with GSH: GSH loss

- Aravindakumar *et al*.* measured loss of GSH with [NO•] >> [GSH]
- pH-Dependence indicated GS^- was reactive form
- Rate constant for GS^- + $NO^{\bullet}$ = 490 M^{-1} s^{-1} at 25°C (effective rate constant ~ 14 M^{-1} s^{-1} at pH 7·4 since [GS^-] ? 3% of $[GSH]_{total}$)
- $t_{½}(NO^{\bullet})$ ~ 10 s with 5 mM GSH at pH 7·4, 25°C

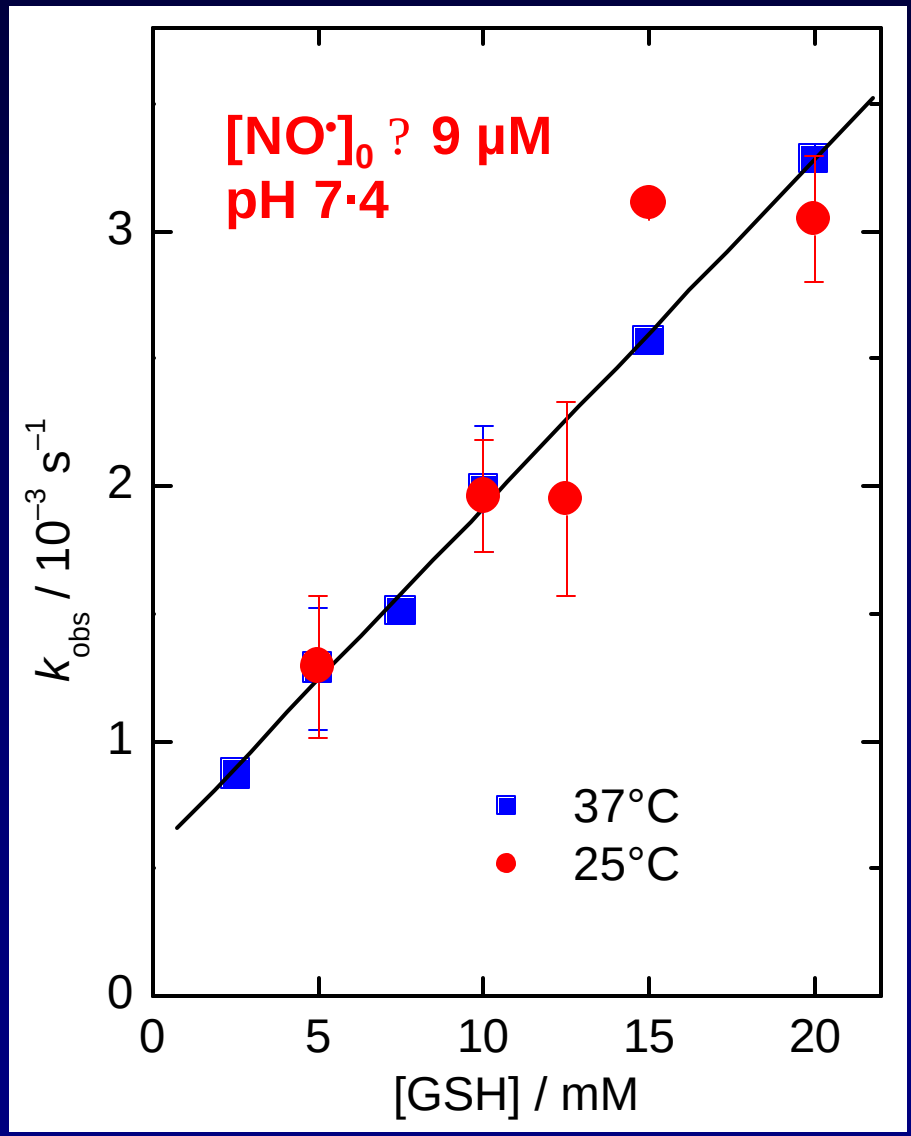


Reactivity ~ *100-fold* faster than suggested from study of Hogg *et al.* (1996)

* *J. Chem. Soc., Perkin Trans. 2*, 663 (2002)

Reaction of $NO^{\bullet}$ with GSH: $NO^{\bullet}$ loss

- Lisa Folkes (Gray Cancer Institute, unpublished) measured $NO^{\bullet}$ loss by chemilumin-escence
- [GSH] >> [$NO^{\bullet}$]
- Results support Hogg *et al.*'s estimate of reactivity
- k ? 0·14 M^{-1} s^{-1} at pH 7·4, 37°C ($t_{½}$($NO^{\bullet}$) ~ 17 min with 5 mM GSH)



Steady-state concentrations

- At a steady-state, **rate of formation = rate of loss**
- Superoxide radicals, no superoxide dismutase:

? $O_2^{\bullet-}$ rate = 0·6 μM s^{-1}

2 $O_2^{\bullet-}$ + 2 H^+ ? H_2O_2 + O_2 rate = 2*k* $[O_2^{\bullet-}]^2$

At steady-state: 6 ? 10^{-7} = 2·4 ? 10^5 $[O_2^{\bullet-}]^2$

$[O_2^{\bullet-}]$? **1·6 μM** (n.b. here '$[O_2^{\bullet-}]$' = $[O_2^{\bullet-}]$ + $[HO_2^{\bullet}]$)

- With 3 μM superoxide dismutase (SOD):

? $O_2^{\bullet-}$ rate = 0·6 μM s^{-1}

$O_2^{\bullet-}$ + SOD ? ½(H_2O_2 + O_2) rate = *k* $[O_2^{\bullet-}]$ [SOD]

At steady-state: 6 ? 10^{-7} = 2·3 ? 10^9 $[O_2^{\bullet-}]$? 3 ? 10^{-6}

$[O_2^{\bullet-}]$? **90 pM**

See: Cadenas & Davies
Free Radical Biol. Med., 2000, **29**, 222

Reversible reactions: driving uphill

- An unfavourable reaction can be driven by removal of a product from the equilibrium
- Le Chatelier's principle (1884, rephrased 1888): 'Every change of one of the factors of an equilibrium occasions a rearrangement of the system … in a sense opposite to the original change.'
- Example: $A + B \underset{k_r}{\overset{k_f}{\rightleftharpoons}} C + D$
  - If forward rate < reverse rate, equilibrium is to left, i.e. if k_f [A] [B] < k_r [C] [D]
  - but if C or D is removed by another reaction, equilibrium can be driven to the right

Product removal can drive an unfavourable equilibrium

- Glutathione often ‘repairs’ drug radicals more efficiently than redox properties predict:

  $drug^{\bullet +} + GSH \rightleftharpoons drug + GS^{\bullet} (+ H^{+})$ $K << 1$

- Drug radical often much weaker oxidant then $GS^{\bullet}$

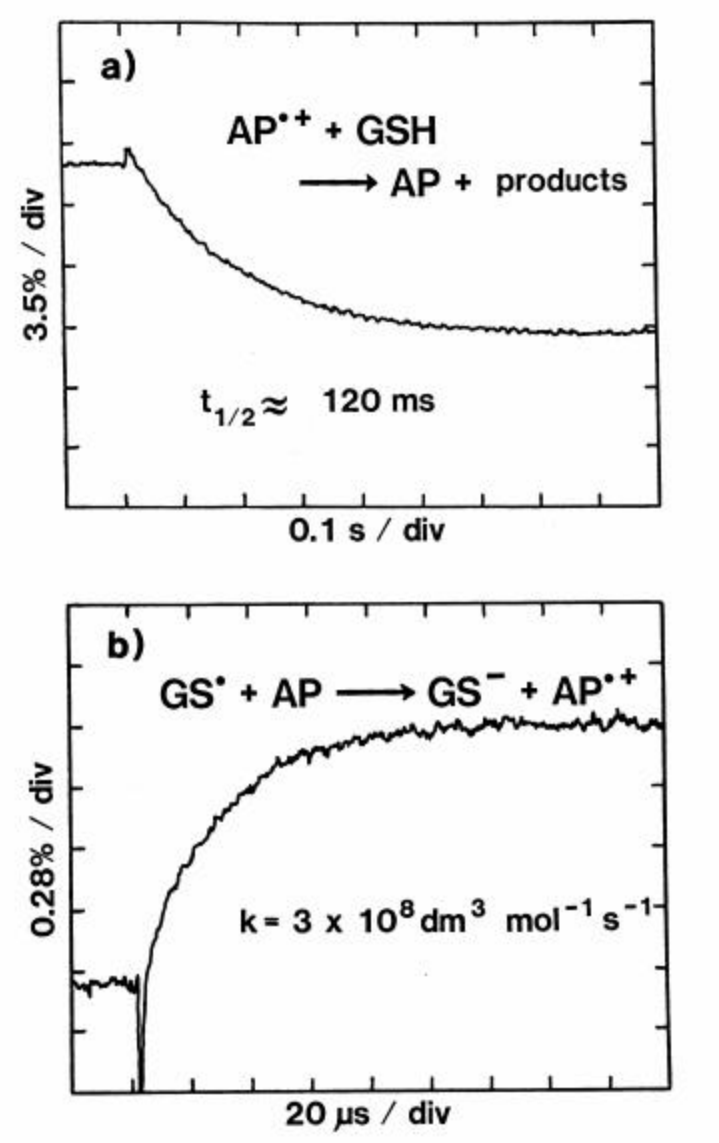
- Removal of product ($GS^{\bullet}$, e.g. by O_2 or ascorbate) drives unfavourable equilibrium to the right

  $GS^{\bullet} + GS^{-} \rightleftharpoons (GSSG)^{\bullet -}$

  $(GSSG)^{\bullet -} + O_2 \rightleftharpoons GSSG + O_2^{\bullet -}$

  $GS^{\bullet} + AscH^{-} \rightleftharpoons GSH + Asc^{\bullet -}$

Unfavourable radical ‘repair’ by GSH

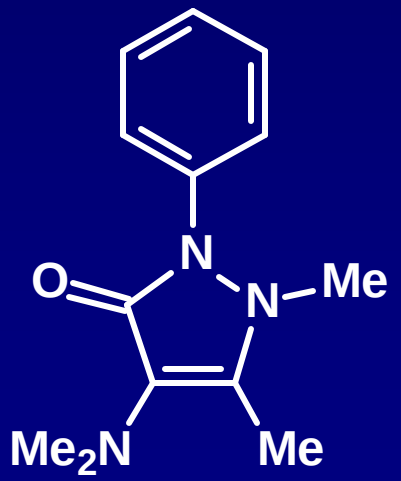


- The radical-cation of aminopyrine (structure below) reacts rapidly with GSH:

$$AP^{\bullet+} + GSH \rightarrow AP + GS^{\bullet} (+ H^{+})$$

- $K < 10^{-4}$ yet reaction proceeds in < 1 s because $GS^{\bullet}$ is removed from the equilibrium

Wilson *et al.*, *Biochem. Pharmacol.*, **35**, 21 (1986)



Lifetimes and diffusion distances

Translating lifetimes to diffusion distances

- Einstein-Smoluchowski equation:

  $\lambda = (6 D t)^{1/2}$

  where λ = root-mean-square diffusion distance in 3-dimensional space

  D = diffusion coefficient

  t = time

- Stokes-Einstein relation:

  $D = kT / 6 \pi \eta a$

  where k = Boltzmann constant

  T = absolute temperature

  η = viscosity

  a = radius of solute species

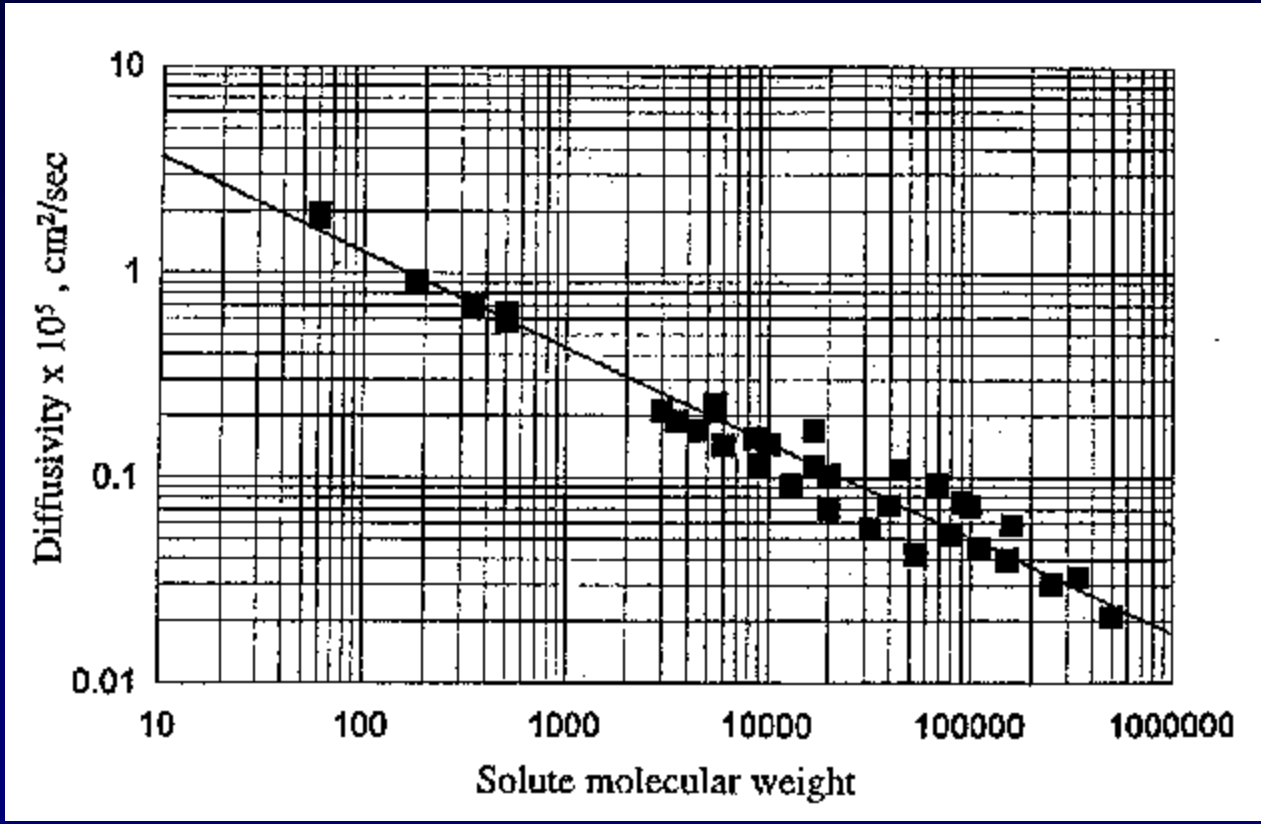
Diffusion coefficients for small molecules

- In water at 25°C (about 25% higher at 37°C)

Solute	*D* / 10^{-9} m^2 s^{-1}	*MW*
$NO^{\bullet}$	3·3	~ 30
O_2	2·4	32
CO_2	1·9	44
$NO_2^{\bullet}$	1·4	46
ethanol	1·2	46
glycine	1·1	75
glucose	0·7	180
sucrose	0·5	342

- Viscosity of blood plasma ~ 1·6 ? that of water
- Viscosity of cytosol may be ~ 1·2 – 4 ? water

Diffusion coefficients for large molecules

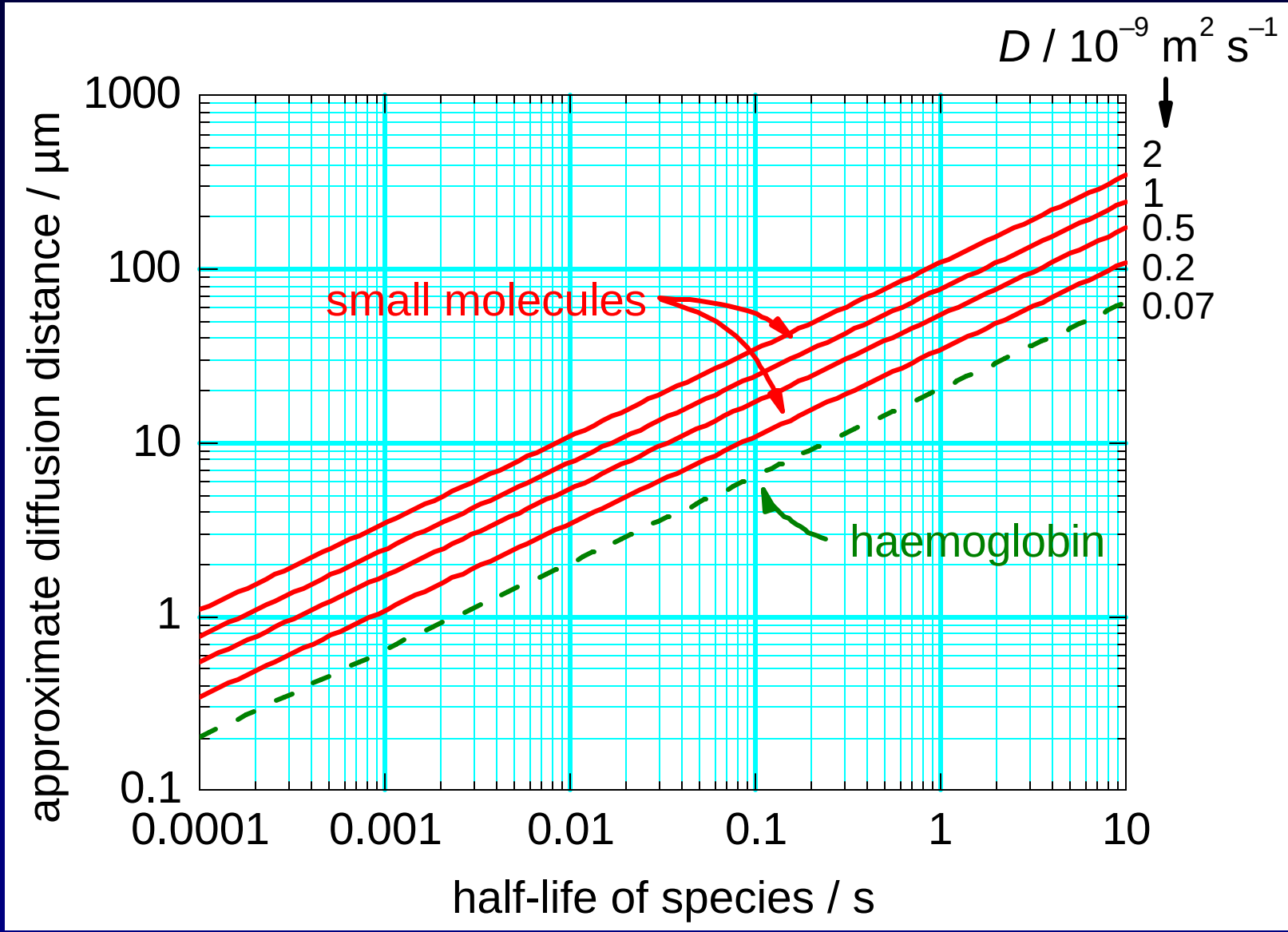


Haemoglobin (~68 kDa):
$D \sim 7 \times 10^{-11}$ m^2 s^{-1}

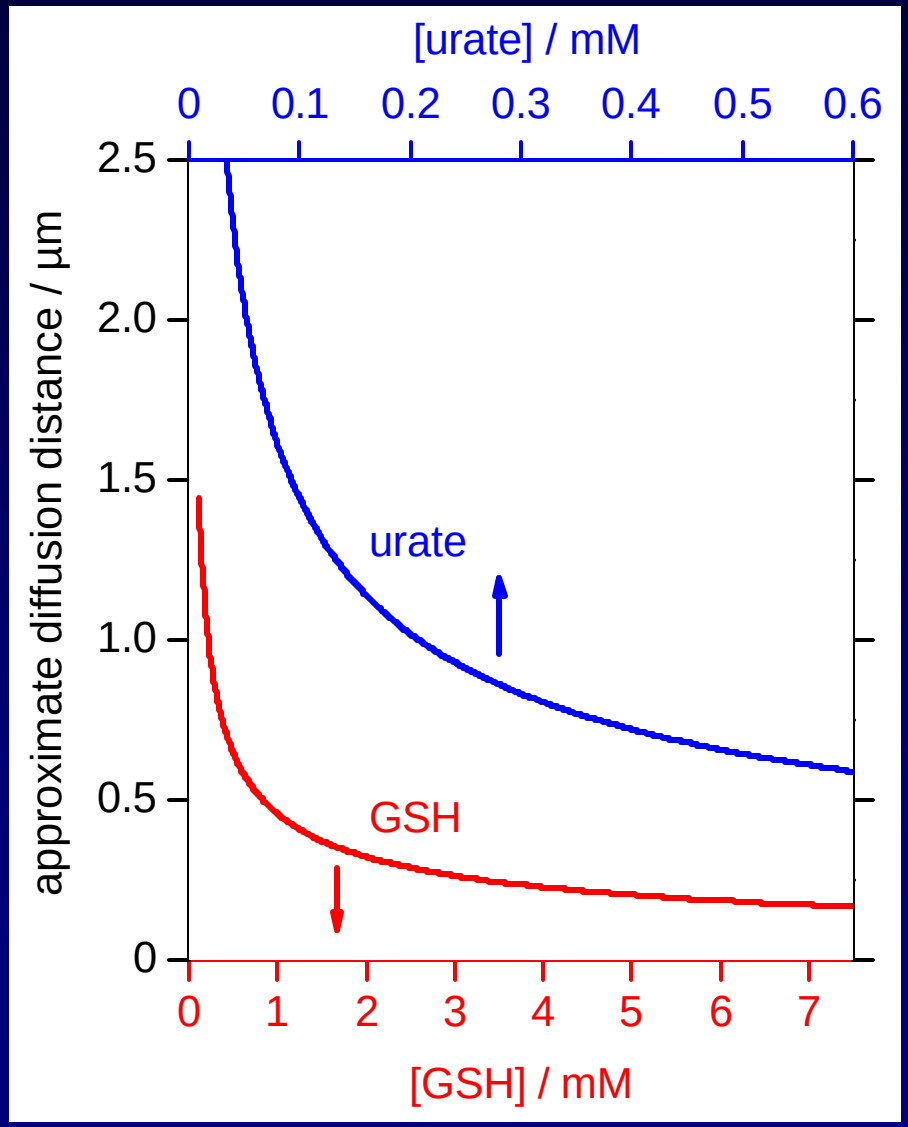
$D = 1{\cdot}0 \times 10^{-8} M^{-0.46}$ m^2 s^{-1} (water, 37°C)
where M is the molecular weight

Fournier, R. L., 1999, *Basic Transport Phenomena in Biomedical Engineering* (Taylor & Francis, Philadelphia)

Approximate diffusion distances



Diffusion of a highly-reactive radical: $NO_2^{\bullet}$



- D ~ 1 ? 10^{-9} m^2 s^{-1}
- k ~ 2 ? 10^7 M^{-1} s^{-1} for both GSH and urate at pH ~ 7·4
  - If [GSH] ~ 5 mM, □ ~ 0·2 µm
  - If [urate] ~ 0·3 mM, □ ~ 0·8 µm
- *All* reactants define $t_{½}$
  - $t_{½}$ ~ 0·7 / ? (k[scavenger])

Mapping oxygen, and protein/protein interactions

Lifetimes of excited states as probes

- Two competing reactions (P* = probe excited state):
  - P* ? light emitted — lifetime of P* = ?? = $1/k_0$
  - P* + Q ? quenching (no light) — rate constant = k_q
- Can measure emission intensity *I* from P*:

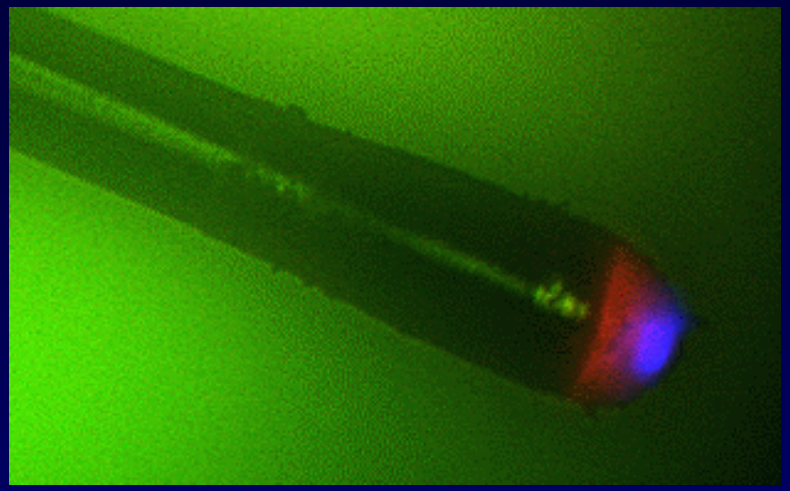
$$I \propto \frac{k_0\,[P^*]}{k_0\,[P^*] + k_q\,[P^*]\,[Q]}$$

$$I_0 / I = \tau_0/\tau = 1 + k_q \tau_0\,[Q]$$

(Stern-Volmer plot – *cf.* competition kinetics)

- **Lifetime imaging** can yield more information
  - may be independent of [P], optical artefacts
  - useful with multi-photon excitation/confocal imaging

Mapping oxygen gradients

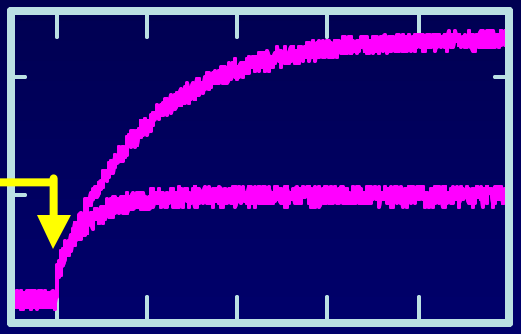


- Optical fibre (200 µm) with $Ru(Ph_2phen)_3$ in silicone rubber coated tip
- Blue light-emitting diode:
  excitation ~ 450 nm
  emission ~ 620 nm

(Borivoj Vojnovic, William K Young & Peter Wardman, Gray Cancer Institute)

- Oxygen-sensitive **lifetime** of luminescence from ruthenium complex measured

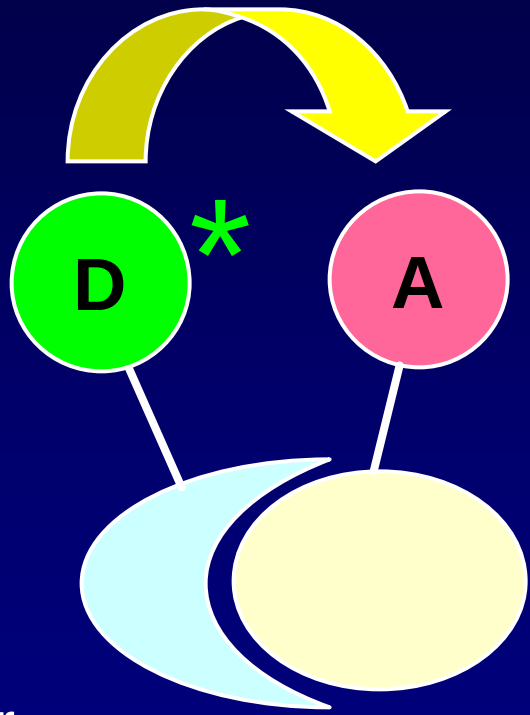
diode switch-on



www.oxfordoptronix.com

Energy transfer between excited states

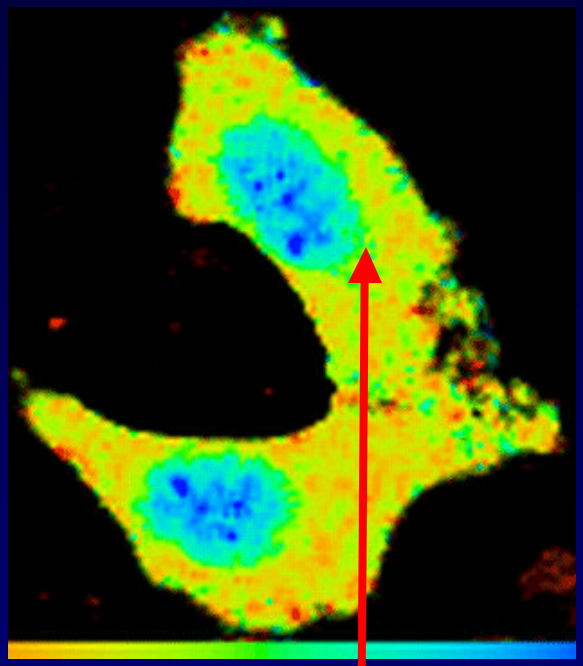
- Tag two (or more) proteins
  - excite donor fluorophor (D)
  - monitor acceptor (A) emission or preferably **lifetime** of donor
  - energy transfer only occurs when proteins interact, reduces lifetime
- **F**luorescence (Förster) **R**esonance **E**nergy **T**ransfer occurs:
  - if excitation spectrum of acceptor overlaps emission spectrum of donor
  - if D/A in close proximity: signal falls off as 1/(distance)^{6} **– 1 to 10 nm range**



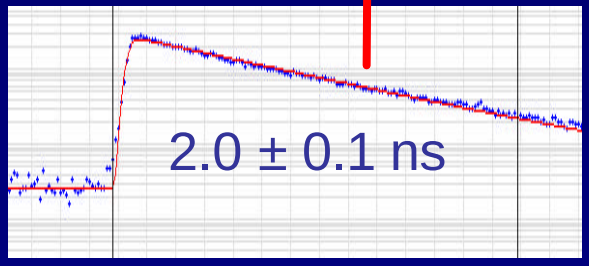
Lifetime imaging: protein interactions

- Tumour cells transfected with NF?B with donor fluorophor tag and antibody-recognisable IKK?
- Cells treated with H_2O_2 and antibodies for IKK? with acceptor fluorophor
- Intensity image shows NF?B distibuted throughout nucleus
- **Lifetime image** (top right) shows oxidative stress activates interaction of NF?B kinase with signalling kinase IKK?, but only in cytoplasm

(Sarah Roberts, Simon Ameer-Beg & Borivoj Vojnovic, Gray Cancer Institute)



1·8 ns **2·4 ns**



donor fluorescence lifetime

Conclusions

THINK about and Use
Thermodynamics and Kinetics
For Understanding and Success

- Perhaps the single most useful kinetic relationship, for reactions (most) where target concentrations significantly exceed radical concentrations, is:

$$\text{radical half-life} \approx \frac{0.7}{\text{sum of (rate constant} \times \text{target concentration)}}$$